

July 6, 2000

**UTILITY PATENT APPLICATION TRANSMITTAL**

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First Named Inventor: Leonard A. SMITH et al.  
Title: RECOMBINANT VACCINE AGAINST BOTULINUM NEUROTOXIN

TO: **BOX PATENT APPLICATION**  
Commissioner for Patents  
Washington, D.C. 20231

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1. ☐ Fee Transmittal Form (original and duplicate)
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CLAIMS AS FILED						
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Total Claims	0	20	0	\$ 18.00	\$ 9.00	\$ 0.00
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First Presentation of Multiple Dependent Claims				\$ 260.00	\$ 130.00	\$ 0.00
<b>BASIC FEE</b>				\$ 690.00	\$ 345.00	\$ 0.00
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3. ☒ Drawings - Total Sheets: 22 (Fig(s). 1-22)
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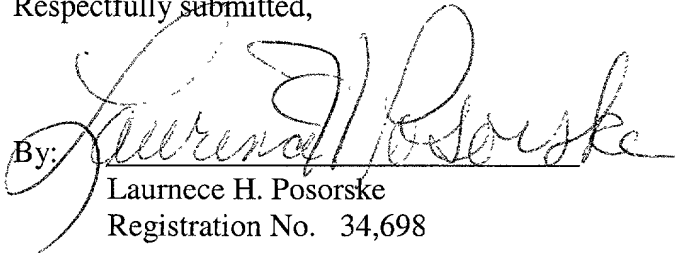
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Prior Application No.: PCT/US00/12890 filed May 12, 2000

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- ☒ Complete Application Based on Provisional Application Nos.: 60/133,866, 60/133,868, 60/133,869, 60/133,865, 60/133,873, 60/133,867 all filed May 12, 1999 and 60/146,192, filed July 29, 1999

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Enclosures

## Recombinant Vaccine Against Botulinum Neurotoxin

This application is based on U.S. Provisional Application Nos. 60/133,866, 60/133,868, 60/133,869, 60/133,865, 60/133,873, 60/133,867, all filed May 12, 1999, and U.S. Provisional Application No. 60/146,192, filed July 29, 1999, all of which are incorporated herein in their entirety.

### BACKGROUND OF THE INVENTION

#### Field of the Invention

This invention is directed to preparation and expression of synthetic genes encoding polypeptides containing protective epitopes of botulinum neurotoxin (BoNT). The invention is also directed to methods of vaccination against botulism using the expressed peptides.

#### Related Art

The sporulating, obligate anaerobic, gram-positive bacillus *Clostridium* produces eight forms of antigenically distinct exotoxins. Tetanus neurotoxin (TeNT) is produced by *Clostridium tetani* while *Clostridium botulinum* produces seven different neurotoxins which are differentiated serologically by specific neutralization. The botulinum neurotoxins (BoNT) have been designated as serotypes A, B, C<sub>1</sub>, D, E, F, and G. Botulinum neurotoxins (BoNT) are the most toxic substances known and are the causative agents of the disease botulism. BoNT exert their action by inhibiting the release of the neurotransmitter acetylcholine at the neuromuscular junction (Habermann, E., et al., (1986), "Clostridial Neurotoxins: Handling and Action at the Cellular and Molecular Level," *Cur. Top. Microbiol. Immunol.*, **129**:93-179; Schiavo, G., et al., (1992a), "Tetanus and Botulinum-B Neurotoxins Block Neurotransmitter Release by Proteolytic Cleavage of Synaptobrevin," *Nature*, **359**:832-835; Simpson, L.L., (1986), "Molecular Pharmacology of Botulinum Toxin and Tetanus Toxin," *Annu. Rev. Pharmacol. Toxicol.*, **26**:427-453) which leads to a state of flaccid paralysis. Indeed, only a few molecules of toxin can abolish the action of a nerve cell. Polyclonal antibodies derived for a specific neurotoxin can neutralize the toxic effects of that toxin but will not cross-neutralize another toxin serotype. Thus, to protect against all seven toxins, one needs seven vaccines.

Botulinum neurotoxins are translated as a single 150 kDa polypeptide chain and then posttranslationally nicked, forming a dichain consisting of a 100 kDa heavy chain and a 50 kDa light chain which remain linked by a disulfide bond (DasGupta, B.R., et al., (1972), "A Common Subunit Structure in *Clostridium botulinum* Type A, B, and E Toxins," *Biophys. Res. Commun.*, **48**:108-112; DasGupta, B.R., (1989), "The Structure of Botulinum Neurotoxins," *Botulinum Neurotoxin and Tetanus Toxin*, (Simpson, L.L., Ed.), pp. 53-67, Academic Press, New York). Most of the clostridial strains contain specific endogenous proteases which activate the toxins at a protease-sensitive loop located approximately one third of the way into the molecule from the amino-terminal end. Upon reduction and fractionation (electrophoretically or chromatographically), the two chains can be separated; one chain has a Mr of ~100 kDa and is referred to as the heavy chain while the other has a Mr ~50 kDa and is termed the light chain.

The mechanism of nerve intoxication is accomplished through the interplay of three key events, each of which is performed by a separate portion of the neurotoxin protein. First, the carboxy half of the heavy chain (fragment C or H<sub>C</sub> is required for receptor specific binding to cholinergic nerve cells (Black, J.D., et al., (1986), "Interaction of <sup>125</sup>I-botulinum Neurotoxins with Nerve Terminals. I. Ultrastructural Autoradiographic Localization and Quantitation of Distinct Membrane Acceptors for Types A and B on Motor Nerves," *J. Cell Biol.*, **103**:521-534; Nishiki, T.-I., et al., (1994), "Identification of Protein Receptor for *Clostridium botulinum* Type B Neurotoxin in Rat Brain Synaptosomes," *J. Biol. Chem.*, **269**:10498-10503; Shone, C.C., et al., (1985), "Inactivation of *Clostridium botulinum* Type A Neurotoxin by Trypsin and Purification of Two Tryptic Fragments. Proteolytic Action Near the COOH-terminus of the Heavy Subunit Destroys Toxin-Binding Activity, *Eur. J. Biochem.*, **151**:75-82). There is evidence suggesting that polysialogangliosides (van Heyningen, W.E., (1968), "Tetanus," *Sci. Am.*, **218**:69-77) could act as receptors for the toxins but the data supporting a specific receptor remains equivocal (Middlebrook, J.L., (1989), "Cell Surface Receptors for Protein Toxins," *Botulinum Neurotoxins and Tetanus Toxin*, (Simpson, L.L., Ed.) pp. 95-119, Academic Press, New York). After binding, the toxin is internalized into an endosome through receptor-mediated endocytosis

(Shone, C.C., et al., (1987), "A 50-kDa Fragment from the NH<sub>2</sub>-terminus of the Heavy Subunit of *Clostridium botulinum* Type A Neurotoxin Forms Channels in Lipid Vesicles, *Euro. J. Biochem.*, **167**:175-180). The amino terminal half of the heavy chain is believed to participate in the translocation mechanism of the light chain across the endosomal membrane (Simpson, 1986; Poulain, B., et al., (1991), "Heterologous Combinations of Heavy and Light Chains from Botulinum Neurotoxin A and Tetanus Toxin Inhibit Neurotransmitter Release in *Aplysia*," *J. Biol. Chem.*, **266**:9580-9585; Montal, M.S., et al., (1992), "Identification of an Ion Channel-Forming Motif in the Primary Structure of Tetanus and Botulinum Neurotoxins," *FEBS*, **313**:12-18). The low pH environment of the endosome may trigger a conformational change in the translocation domain, thus forming a channel for the light chain. The final event of intoxication involves enzymatic activity of the light chain, a zinc-dependent endoprotease (Schiavo, 1992a; Schiavo, G., et al., (1992b), "Tetanus Toxin is a Zinc Protein and its Inhibition of Neurotransmitter Release and Protease Activity Depend on Zinc," *EMBO J.*, **11**:3577-3583), on key synaptic vesicle proteins (Schiavo, 1992a; Oguma, K., et al., (1995), "Structure and Function of *Clostridium botulinum* Toxins," *Microbiol. Immunol.*, **39**:161-168; Schiavo, G., et al., (1993), "Identification of the Nerve Terminal Targets of Botulinum Neurotoxin Serotypes A, D, and E," *J. Biol. Chem.*, **268**:23784-23787; Shone, C.C., et al., (1993), "Proteolytic Cleavage of Synthetic Fragments of Vesicle-Associated Membrane Protein, Isoform-2 by Botulinum Type B Neurotoxin," *Eur. J. Biochem.*, **217**:965-971) necessary for neurotransmitter release. The light chains of BoNT serotypes A, C<sub>1</sub>, and E cleave SNAP-25 (synaptosomal-associated protein of M25,000), serotypes B, D, F, and G cleave VAMP/synaptobrevin (synaptic vesicle-associated membrane protein); and serotype C<sub>1</sub> cleaves syntaxin. Inactivation of SNAP-25, VAMP, or syntaxin by BoNT leads to an inability of the nerve cells to release acetylcholine resulting in neuromuscular paralysis and possible death, if the condition remains untreated.

Human botulism poisoning is generally caused by type A, B, E or rarely, by type F toxin. Type A and B are highly poisonous proteins which resist digestion by the enzymes of the gastrointestinal tract. Foodborne botulism poisoning is caused by the toxins present in contaminated food, but wound and infant botulism are caused

by in vivo growth in closed wounds and the gastrointestinal tract respectively. The toxins primarily act by inhibiting the neurotransmitter acetylcholine at the neuromuscular junction, causing paralysis. Another means for botulism poisoning to occur is the deliberate introduction of the toxin(s) into the environment as might  
5 occur in biological warfare. When the cause of botulism is produced by toxin rather than by *in vivo* infection the onset of neurologic symptoms is usually abrupt and occurs within 18 to 36 hours after ingestion. The most common immediate cause of death in respiratory failure due to diaphragmatic paralysis. Home canned foods are the most common sources of toxins. The most frequently implicated toxin is toxin  
10 A, which is responsible for more than 50% of morbidity resulting from botulinum toxin.

Because even small amounts of botulinal toxin can cause serious illness, persons such as laboratory workers who are exposed to toxin must learn to handle all samples that may contain toxin with extreme care. It is also suggested that such  
15 workers be protected from illness by vaccination against the toxins. Furthermore, persons exposed to conditions in which botulism toxins might be in the environment which might be inhaled or ingested, such as military personnel, need to be protected from the toxin.

Agents that abolish the action of BoNT have been investigated since the  
20 1940s. Early work at Fort Detrick in the 1940s lead to the development of a toxoid vaccine to protect against serotypes A, B, C<sub>1</sub>, D, and E toxins. The toxoid vaccine was manufactured by growing five *Clostridium botulinum* strains, extracting and precipitating the toxin from the growth media after cell lysis. Formalin was added to the crude preparation to inactivate the neurotoxin. Residual formalin was left in the  
25 vaccine product to ensure the toxin remains non-toxic. The product was adsorbed to aluminum hydroxide and blended. Currently, a pentavalent toxoid vaccine against serotypes A through E (Anderson, J.H., et al., (1981), "Clinical Evaluation of Botulinum Toxoids," *Biomedical Aspects of Botulism*, (Lewis, G.E., Ed.), pp. 233-246, Academic Press, New York; Ellis, R. J., (1982), "Immunobiologic Agents and  
30 Drugs Available from the Centers for Disease Control. Descriptions, Recommendations, Adverse Reactions and Scrologic Response," 3rd ed., Centers for Disease Control. Atlanta, GA; Fiock, M.A., et al., (1963), "Studies of Immunities to

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Toxins of *Clostridium Botulinum*. IX. Immunologic Response of Man to Purified Pentavalent ABCDE Botulinum Toxoid," *J. Immunol.*, **90**:697-702; Siegel, L.S., (1988), "Human Immune Response to Botulinum Pentavalent (ABCDE) Toxoid Determined by a Neutralization Test and by an Enzyme-Linked Immunosorbent Assay," *J. Clin. Microbiol.*, **26**:2351-2356), available under Investigational New Drug (IND) status, is used to immunize specific populations of at-risk individuals, i.e., scientists and health care providers who handle BoNT and our armed forces who may be subjected to weaponized forms of the toxin. Though serotypes A, B, and E are most associated with botulism outbreaks in humans, type F has also been diagnosed (Midura, T.F., et al., (1972), "*Clostridium botulinum* Type F: Isolation from Venison Jerky," *Appl. Microbiol.*, **24**:165-167; Green, J., et al., (1983), "Human Botulism (Type F) – A Rare Type," *Am. J. Med.*, **75**:893-895; Sonnabend, W.F., et al., (1987), "Intestinal Toxicoinfection by *Clostridium botulinum* Type F in an Adult. Case Associated with Guillian-Barre Syndrome," *Lancet*, **1**:357-361; Hatheway, C.L., (1976), "Toxoid of *Clostridium botulinum* Type F: Purification and Immunogenicity Studies," *Appl. Environ. Microbiol.*, **31**:234-242). A separate monovalent toxoid vaccine against BoNTF is available under IND status. Hatheway demonstrated that the BoNTF toxoid could protect guinea pigs against a homologous challenge (Wadsworth, J.D.F., et al., (1990), "Botulinum Type F Neurotoxin," *Biochem. J.*, **268**:123-128).

Even though toxoid vaccines are available, there are numerous shortcomings with their current use and ease of production. First, because *C. botulinum* is a spore-former, a dedicated facility is required to manufacture a toxin-based product. The requirement for a dedicated manufacturing facility is not trivial. It is extremely costly to renovate and upgrade an existing facility or to build a new one and then to maintain the facility in accordance with current Good Manufacturing Practices (cGMP) to manufacture one vaccine. Second, the yields of toxin production from *C. botulinum* are relatively low. Third, the toxoiding process involves handling large quantities of toxin and thus is dangerous, and the added safety precautions increase the cost of manufacturing. Fourth, the toxoid product for types A-E consists of a crude extract of clostridial proteins that may influence immunogenicity or reactivity of the vaccine, and the type F toxoid is only partially purified (IND 5077). Fifth,



because the toxoiding process involves the use of formaldehyde, which inactivates the toxin, and residual levels of formaldehyde (not to exceed 0.02%) are part of the product formulation to prevent reactivation of the toxin, the vaccine is reactogenic. An additional component of the toxoid vaccines is the preservative thimerosal (0.01%), which also increases the reactogenicity of the product.

The development of a new-generation, recombinant vaccine could alleviate many of the problems associated with the toxoid. A recombinant vaccine would eliminate the need for a dedicated manufacturing facility. Presently, many cGMP facilities are in existence and available that could manufacture a recombinant product. There would be no need to culture large quantities of a hazardous toxin-producing bacterium. Production yields from a genetically engineered product is expected to be high. There would be no need to treat the vaccine with formalin because the product would be non-toxic from the outset. Recombinant products would be purer, less reactogenic, and more fully characterized. Thus, the cost of a recombinant product would be expected to be much lower than a toxoid because there would be no expenditures required to support a dedicated facility, and the higher production yields would reduce the cost of the vaccine product.

#### SUMMARY OF THE INVENTION

It is an object of this invention to provide immunogenic peptides capable of eliciting protective immunity against botulinum neurotoxin of serotypes A-G.

It is another object of this invention to provide vaccines capable of eliciting protective immunity against botulinum neurotoxin, where the vaccines do not act as neurotoxins themselves.

It is yet another object of this invention to provide methods for preparing non-toxic peptides for use in vaccines against botulinum neurotoxin by growing recombinant organisms which express the peptides.

It is still another object of this invention to provide methods for fast and efficient purification of the non-toxic peptides from cultures of recombinant organisms.

These and other objects are met by one or more of the following embodiments of the present invention

In one embodiment, this invention provides a nucleic acid encoding the carboxy-terminal portion of the heavy chain (HC) of botulinum neurotoxin (BoNT), the BoNT being selected from the group consisting of BoNT serotype A, BoNT serotype B, BoNT serotype C1, BoNT serotype D, BoNT serotype E, BoNT serotype F, and BoNT serotype G, wherein said nucleic acid is expressable in a recombinant organism selected from *Escherichia coli* and *Pichia pastoris*. Preferably, the nucleic acid comprises a nucleic acid sequence selected from SEQ ID No:1 (serotype A), SEQ ID No:7 (serotype B), SEQ ID No:9 (serotype C1), SEQ ID No:11 (serotype D), SEQ ID No:13 (serotype E), SEQ ID No:15 (serotype F), and SEQ ID No:17 (serotype G). In an alternative preferred embodiment, the nucleic acid encodes an HC amino acid sequence of BoNT selected from SEQ ID No:2 (serotype A), SEQ ID No:8 (serotype B), SEQ ID No:10 (serotype C1), SEQ ID No:12 (serotype D), SEQ ID No:14 (serotype E), SEQ ID No:16 (serotype F), and SEQ ID No:18 (serotype G).

In another embodiment, this invention provides a nucleic acid encoding the amino-terminal portion of the heavy chain (HN) of botulinum neurotoxin (BoNT), the BoNT being selected from the group consisting of BoNT serotype B, BoNT serotype C1, BoNT serotype D, BoNT serotype E, BoNT serotype F, and BoNT serotype G, wherein said nucleic acid is expressable in a recombinant organism selected from *Escherichia coli* and *Pichia pastoris*. In a preferred embodiment, the nucleic acid comprises a nucleic acid sequence selected from SEQ ID No:21 (serotype B), SEQ ID No:23 (serotype C1), SEQ ID No:25 (serotype D), SEQ ID No:27 (serotype E), SEQ ID No:29 (serotype F), and SEQ ID No:31 (serotype G). Alternatively, the nucleic acid encodes an HN amino acid sequence of BoNT selected from SEQ ID No:22 (serotype B), SEQ ID No:24 (serotype C1), SEQ ID No:26 (serotype D), SEQ ID No:28 (serotype E), SEQ ID No:30 (serotype F), and SEQ ID No:32 (serotype G).

Preferably, the nucleic acid of this invention is a synthetic nucleic acid. In a preferred embodiment, the sequence of the nucleic acid is designed by selecting at least a portion of the codons encoding HC from codons preferred for expression in a host organism, which may be selected from gram negative bacteria, yeast, and mammalian cell lines; preferably, the host organism is *Escherichia coli* or *Pichia*

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pastoris. In another preferred embodiment, the nucleic acid sequence encoding HC is designed by selecting codons encoding HC which codons provide HC sequence enriched in guanosine and cytosine residues. More preferably, nucleic acid encoding HC or HN is expressed in a recombinant host organism with higher yield than a second nucleic acid fragment encoding the same HC sequence, said second nucleic acid fragment having the wild-type *Clostridium botulinum* sequence of HC.

In yet another embodiment, this invention provides an expression vector comprising the nucleic acid of this invention, whereby HC and/or HN is expressed upon transfection of a host organism with the expression vector. Another embodiment of this invention provides a method of preparing a polypeptide comprising the carboxy-terminal portion of the heavy chain (HC) of botulinum neurotoxin (BoNT) or the amino-terminal portion of the heavy chain (HN) of botulinum neurotoxin (BoNT) selected from the group consisting of BoNT serotype A, BoNT serotype B, BoNT serotype C, BoNT serotype D, BoNT serotype E, BoNT serotype F, and BoNT serotype G, said method comprising culturing a recombinant host organism transfected with the expression vector of of this invention under conditions wherein HC or HN is expressed. Preferably, the recombinant host organism is a eukaryote. In another preferred embodiment, the method of this invention further comprises recovering insoluble protein from the host organism, whereby a fraction enriched in HC or HN is obtained. Preferably, the host organism is *Pichia pastoris*.

In still another embodiment, this invention provides an immunogenic composition comprising the carboxy-terminal portion of the heavy chain (HC) of botulinum neurotoxin (BoNT) selected from the group consisting of BoNT serotype A, BoNT serotype B, BoNT serotype C, BoNT serotype D, BoNT serotype E, BoNT serotype F, and BoNT serotype G. Preferably, the immunogenic composition is prepared by culturing a recombinant organism transfected with an expression vector encoding HC. More preferably, the immunogenic composition is prepared by a method wherein an insoluble protein fraction enriched in HC is recovered from said recombinant organism.

In yet another embodiment, this invention provides an immunogenic composition comprising the amino-terminal portion of the heavy chain (HN) of

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botulinum neurotoxin (BoNT) selected from the group consisting of BoNT serotype A, BoNT serotype B, BoNT serotype C, BoNT serotype D, BoNT serotype E, BoNT serotype F, and BoNT serotype G. Preferably, the immunogenic composition comprising HN is prepared by culturing a recombinant organism transfected with an expression vector encoding HN. More preferably, the immunogenic composition is prepared from an insoluble protein fraction enriched in HN which is recovered from the recombinant organism.

In still another embodiment, this invention provides an immunogenic composition comprising a polypeptide comprising epitopes contained in the carboxy-terminal portion of the heavy chain (HC) of botulinum neurotoxin (BoNT) and/or the amino-terminal portion of the heavy chain (HN) of botulinum neurotoxin (BoNT) selected from the group consisting of BoNT serotype A, BoNT serotype B, BoNT serotype C, BoNT serotype D, BoNT serotype E, BoNT serotype F, and/or BoNT serotype G, said epitopes eliciting protective immunity toward the respective BoNT serotype. Preferably, the immunogenic composition elicits an ELISA response to the respective BoNT serotype(s) in an animal which is detectable in serum from the animal even when the serum is diluted 100-fold.

#### **BRIEF DESCRIPTION OF THE DRAWINGS**

Figure 1 shows the sequence for a synthetic gene encoding the HC fragment of BoNT serotype A and the encoded amino acids sequence.

Figure 2 shows the sequence for a synthetic gene encoding the HC fragment of BoNT serotype A and the encoded amino acids sequence.

Figure 3 shows the sequence for a synthetic gene encoding the HC fragment of BoNT serotype A and the encoded amino acids sequence.

Figure 4 shows the sequence for a synthetic gene encoding the HC fragment of BoNT serotype B and the encoded amino acids sequence.

Figure 5 shows the sequence for a synthetic gene encoding the HC fragment of BoNT serotype C and the encoded amino acids sequence.

Figure 6 shows the sequence for a synthetic gene encoding the HC fragment of BoNT serotype D and the encoded amino acids sequence.

Figure 7 shows the sequence for a synthetic gene encoding the H<sub>C</sub> fragment of BoNT serotype E and the encoded amino acids sequence.

Figure 8 shows the sequence for a synthetic gene encoding the H<sub>C</sub> fragment of BoNT serotype E and the encoded amino acids sequence.

5        Figure 9 shows the sequence for a synthetic gene encoding the H<sub>C</sub> fragment of BoNT serotype F and the encoded amino acids sequence.

Figure 10 shows the sequence for a synthetic gene encoding the H<sub>C</sub> fragment of BoNT serotype G and the encoded amino acids sequence.

10       Figure 11 shows the sequence for a synthetic gene encoding the H<sub>N</sub> fragment of BoNT serotype A and the encoded amino acids sequence.

Figure 12 shows the sequence for a synthetic gene encoding the H<sub>N</sub> fragment of BoNT serotype B and the encoded amino acids sequence.

Figure 13 shows the sequence for a synthetic gene encoding the H<sub>N</sub> fragment of BoNT serotype C and the encoded amino acids sequence.

15       Figure 14 shows the sequence for a synthetic gene encoding the H<sub>N</sub> fragment of BoNT serotype D and the encoded amino acids sequence.

Figure 15 shows the sequence for a synthetic gene encoding the H<sub>N</sub> fragment of BoNT serotype E and the encoded amino acids sequence.

20       Figure 16 shows the sequence for a synthetic gene encoding the H<sub>N</sub> fragment of BoNT serotype F and the encoded amino acids sequence.

Figure 17 shows the sequence for a synthetic gene encoding the H<sub>N</sub> fragment of BoNT serotype G and the encoded amino acids sequence.

Figure 18 shows the sequence for a synthetic gene encoding the H<sub>C</sub> fragment of BoNT serotype F and the encoded amino acids sequence.

25       Figure 19 shows (A) AT base content of a putative fragment C region in native C. botulinum DNA. (B) Reduction at AT content after the first design (rBoNTF(Hc)1) of the synthetic gene. (C) AT content of the final gene design (rBoNTF(Hc)2) used to express recombinant rBoNTF(Hc) in P. pastoris.

30       Figure 20 shows (A) SDS-PAGE and (B) Western blot of samples at various steps along the rBoNTF(Hc) purification. Lanes from both figures are identical except lane 1, where SDS-PAGE shows Novex mark 12 wide-range molecular weight markers and Western blot shows Novex See Blue prestained molecular

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weight markers. Lane 2 is the cell lysate, lane 3 is the cell extract, lane 4 is the cell extract after dialysis, lane 5 is pool of rBoNTF(Hc) positive fractions after Mono S column chromatography, and lane 6 is pool of rBoNTF(Hc)-positive fractions after hydrophobic interaction chromatography.

5        Figure 21 shows purification of rBoNTF(Hc) by sequential chromatography. (A) Mono S cation exchange chromatography of extract from *P. pastoris*. Proteins were eluted with increasing NaCl gradient. Fractions positive for rBoNTF(Hc) by Western analysis were pooled individually and subjected to hydrophobic interaction chromatography (B) and proteins were eluted with a decreasing ammonium sulfate  
10        gradient. In both panels, protein monitored by A280nm is recorded on the left axis and elution conditions are recorded on the right axis, with the gradient trace laid over the chromatogram.

          Figure 22 shows CD spectra of purified soluble (—) and resolubilized (—) rBoNTF(Hc) at 30  $\mu$ g/ml (0.62  $\mu$ M) in 10 mM sodium phosphate, pH 7.0 in a 1-cm  
15        path length cell. Spectra were the average of four accumulations, scanned from 260 to 200 nm at a scan rate of 10 nm/min with a 2-s response and a 1-nm bandwidth. The temperature was maintained at 20°C using a Peltier thermocontrol device.

#### 20        DETAILED DESCRIPTION OF THE EMBODIMENTS OF THIS INVENTION

          The present inventors have determined that animals, including primates, may be protected from the effects of botulinum neurotoxin (BoNT) by immunization with fragments of the botulinum neurotoxin protein expressed by recombinant organisms. Specifically, peptides comprising protective epitopes from the receptor  
25        binding domain and/or the translocation domain, found in the carboxy terminal and the amino terminal portions of the heavy chain of the BoNT protein, respectively, are expressed by recombinant organisms transfected with expression vectors encoding the peptides for each serotype of BoNT. Immunization with these recombinantly produced peptides will elicit antibodies capable of protecting animals  
30        against intoxication with the BoNT of the respective serotype.

          This invention provides a genetically engineered vaccine for protection against botulism. The vaccine comprises fragments of the A and B toxins known as

"C fragments" (H<sub>C</sub> domain). It is now possible to produce the H<sub>C</sub> fragments of the A and B toxins in *E. coli* using gene segment constructs of the HC fragment or an HC polypeptide fused to *E. coli* maltose binding protein. It has been found that the fusion product provides excellent protection against the native toxin challenge. The invention provides plasmids and recombinant proteins for use as vaccines to provide protection against toxins of *Clostridium botulinum*.

Kozaki et al. in "Antibodies against Botulism Neurotoxin", L.L. Simpson, ed., 1989, Academic Press, New York, suggested that a protective epitope might be present in the 50 kDa carboxyl terminus (HC) region of the protein. Thompson et al. (1990, *Eur. J. Biochem.* **189**:73-81) deduced the amino acid sequence for the serotype A botulinum toxin. DasGupta, et al. (1990, *Biochemie*, **72**:661-664), identified the "nick" site for post-translational cleavage of the expressed toxin polypeptide, from which the sequence of the heavy chain can be deduced as follows (see also Krieglstein, et al., 1994, *J. Protein Chem.*, **13**:49-57):

ALNDLCIKVNNWDLFFSPSEDNFTNDLNKGEEITSDTNIEAAEENISL  
DLIQYYLTFNFDNEPENISIEENLSSDIIGQLELMPNIERFPNGKKYELDKYTM  
FHYLRAQEFEGHGSRIALTNSVNEALLNPSRVYTFSSDYVKKVNKATEAA  
MFLGWVEQLVYDFTDETSEVSTTDKIADITIIPYIGPALNIGMLYKDDFVGA  
LIFSGAVILLEFIPEIAIPVLGTFALVSYIANKVLTQVTDNALS KRNEKWDEV  
YKYIVTNWLAKVNTQIDLIRKKMKEALENQAEATKAIINYQYNQYTEEEKN  
NINFNIDDLSSKLNESINKAMININKFLNQCSVSYLMNSMIPYGVKRLEDFDA  
SLKDALLKYIRDNYGTLIGQVDRDKDVNNTLSTDIPFQLSKYVDNQRLST  
FTEYIKNIINTSILNLRYESNHLIDLSRYASKINIGSKVNFDPIDKNQIQLFNLES  
SKIEVILKNAIVYNSMYENFSTSFWRIPKYFNSISLNNEYTIINCMENNSGWK  
VSLNYGEIHWTLQDTQEIKQRVVFYKYSQMINISDYINRWIFVTITNNRLNNSKI  
YTINGRLIDQKPISNLGNIHASNNIMFKLDGCRDTHRYIWIWIKYFNLFDKELNE  
KEIKDLYDNQSN SGILKDFWGDYLQYDKPYMILLYDPNKYVDVNNVGIR  
GYMYLKGPRGSVMTTNIYLNSSLYRGTKFIKKASGNKDNIVRNNDRVYIN  
VVVKNKEYRLATNASQAGVEKILSALEIPDVGNLSQVVVMKSKNDQGITNK  
CKMNLQDNNGNDIGFIGFHQFNNAIKLVASNWYNROTERTSSRTLGCSEWFI  
PVDDGWGERPL

Whelan et al. (*Appl. Environ. Microbiol.* **58**:2345-2354, 1992) have deduced the amino acid sequence for the serotype B botulinum toxin. Schmidt, et al. (1985, *Arch. Biochem. Biophys.*, **238**:544-548), provided N-terminal sequence information for the heavy chain resulting from post-translational cleavage of the expressed toxin polypeptide, and the sequence of the heavy chain can be deduced from this information as follows:

APGICIDVDNEDLFFIADKNSFSDDLKNERIEYNTQSNIYENDFPINEL  
ILDIDLISKIELPSENTESLTDFNVDVPVYEKQPAIKKIFTDENTIFQYLYSQTF  
10 PLDIRDISLTSSFDDALLFSNKVYSFFSMDYIKTANKVVEAGLFAGWVKQIV  
NDFVIEANKSNTMDKIADISLIVPYIGLALNVGNETAKGNFENAFEIAGASIL  
LEFIPELLIPVVGAFLLSYIDNKNKIIKTIDNALTKRNEKWSDMYGLIVAQW  
LSTVNTQFYTIKEGMYKALNYQAQALEEIIKYRYNIYSEKEKSNINIDFNDIN  
SKLNEGINQAIDNINNFINGCSVSYLMKKMIPLAVEKLLDFDNTLKKNLLNYI  
15 DENKLYLIGSAEYEKSKVNKYLKTIMPFDLSTYTNDTILEMFNKYNSEILNNI  
ILNRLRYKDNNLIDLSGYGAKVEVYDGVELNDKNQFKLTSSANSKIRTTQNN  
NIIFNSVFLDFSVSFWIRIPKYKNDGIQNYIHNEYTIINCMKNNSGWKISIRGRI  
IWTLDINGKTKSVFFEYNIREDISEYINRWFFVTITNNLNNAKIYINGKLESN  
TDIKDIREVIANGEIIFKLDGDIDRTQFIWMKYFSIFNTELSQSNIERYKIQSY  
20 SEYLKDFWGNPLMYNKEYYMFNAGNKNSYTIKLLKSDPVGELTRSKYNQ  
NSKYINYRDLYIGEKFIIRRKSNSQSINDDIVRKEDIYLDFFNLNQEWRVYT  
YKYFKKEEELFLAPISDSDEFYNTIQIKEYDEQPTYSCQLLFKKDEESTDEIGL  
IGIHRFYESGIVFEEVKDVFCSIDWYLEVVRKPYNLKLGCNWQFIPKDEGWT  
E

25 Analogous post-translational cleavage for all BoNT serotypes produces analogous heavy chain and light chain structures (see Krieglstein, et al., 1994, *J. Protein Chem.*, **13**:49-57, and references cited therein).

#### **Synthetic Gene Construction**

30 Preliminary experiments indicated that the DNA sequence found in *C. botulinum* encoding the relevant BoNT fragments are not well expressed in typical recombinant hosts. Therefore, synthetic gene construction was undertaken, based on the amino acid sequence of the respective fragments.



Synthetic gene construction is a technique used to optimize for expression in heterologous host systems. The base composition (i.e., percent A+T or percent G+C) as well as the specific codons in a gene sequence play a role in determining whether a gene from one organism will be optimally expressed in a different organism. There is a reason why certain codons are used and why some are not. Organisms will use the codons in which corresponding tRNAs are present. If the organisms do not use certain codons, they most likely lack those specific tRNAs. As it turns out, codons found in clostridial DNA (i.e., genes found in the genus of bacterial called *Clostridium*) are very unique both in terms of base composition (i.e., very high A+T base composition) and in the use of codons not normally found in *E. coli* or yeast.

Table 1 is a chart depicting codon usage in *Pichia pastoris*. This table was generated by listing the codons found in a number of highly expressed genes in *P. pastoris*. The codon data was obtained by sequencing the genes and then listing which codons were found in the genes.

From Table 1, it is clear that the amino acid residues can be encoded for by multiple codons. When constructing synthetic genes using *P. pastoris* codon usage, it is preferred to use only those codons that are found in the naturally occurring genes in *P. pastoris*, and it should be attempted to keep them in the same ratio found in the genes of the natural organism. When the clostridial gene has an overall A+T richness of greater than 70% and A+T regions that have spikes of A+T of 95% or higher, they have to be lowered for expression in expression systems like yeast. (Preferably, the overall A+T richness is lowered below 60% and A+T in spikes is also lowered to 60% or below). It is of course necessary to balance keeping the same codon ratio (e.g., for glycine GGG was not found, GGA was found 22% of the time, GGT was found 74% of the time, GGC was found 3% of the time) with reducing the high A+T content. In the construction of the genes, it is preferred to keep the A+T spikes about 55%.

Considering codon usage for a number of organisms including *E. coli*, it turns out that a synthetic gene using *E. coli* codon usage also expresses fairly well in *P. pastoris*. Similarly, a synthetic gene using *P. pastoris* codon usage also appears to express very well in *E. coli*.

Synthetic genes for the H<sub>C</sub> fragments of botulinum neurotoxin serotypes A-G are shown in Figures 1-10, along with the amino acid sequences encoded by the synthetic genes. Synthetic genes for the H<sub>N</sub> fragments of botulinum neurotoxin serotypes A-G are shown in Figures 11-17, along with the amino acid sequences encoded by the synthetic genes. Synthetic genes having alternative gene sequences may be prepared by following the guidance provided herein concerning codon selection. The amino acid sequence encoded by such synthetic genes will preferably be the sequence of one of the BoNT serotype proteins, or a fragment thereof which contains protective epitopes. Suitable fragments include the H<sub>C</sub> fragments of BoNT serotypes A, B, C<sub>1</sub>, D, E, F, and G, and the H<sub>N</sub> fragments of BoNT serotypes A, B, C<sub>1</sub>, D, E, F, and G. Such alternative gene sequences are within the contemplation of this invention.

Also within the contemplation of this invention are proteins containing protective epitopes from both the N-terminal and the C-terminal domains of the respective serotype BoNT proteins. Such proteins may be prepared by fusing a sequence encoding the translocation domain (HN) to the sequence of the HC region. This may be accomplished by removing the restriction enzyme site of the 3' end of the translocation domain gene as well as the termination codon, and also removing the initiation codon, restriction enzyme site and any other nucleotides on the 5' end of the gene that are not part of the botulinum toxin gene. Then a common restriction enzyme site not found in either synthetic gene may be inserted on the 3' end of the H<sub>N</sub> gene and the 5' end of the H<sub>C</sub> gene, and this common restriction site may be used to fuse the two genes together.

#### **Recombinant Peptide Production**

The nontoxin fragment is very safe, will not require formalin treatment, and has been shown to produce significant immunity against the fully toxic parent molecule. There are two major advantages of the invention over the presently employed vaccine. First, the recombinantly-produced botulinum neurotoxin (rBoNT) protein fragments are completely nontoxic and, is thus, very safe. The fermentation of the host cell harboring the rBoNT gene (e.g., *Escherichia coli* or *Pichia pastoris*) will not require the high biological containment facilities presently needed to ferment the spore-forming *Clostridium botulinum* required for the

production of the toxoid vaccine. Second, the synthetic gene can be placed in high expression systems and used to make much larger quantities of the fragment than toxin produced by the parent organism, *Clostridium botulinum*. Thus, there will be immense cost savings because it will be easier and safer to produce much larger quantities of the vaccine than is now possible.

Synthetic genes as described herein may be transfected into suitable host organisms to create recombinant production organisms, and cultures of these recombinant organisms can then be used to produce immunogenic peptide fragments capable of conferring protective immunity against BoNT of the respective serotypes.

Exemplary techniques for transfection and production of BoNT fragments are shown in the Examples. Alternative techniques are well documented in the literature (See, e.g., Maniatis, Fritsch & Sambrook, "Molecular Cloning: A Laboratory Manual" (1982); "DNA Cloning: A Practical Approach," Volumes I and II (D.N. Glover, ed., 1985); "Oligonucleotide Synthesis" (M.J. Gait, ed., 1984); "Nucleic Acid Hybridization" (B.D. Hames & S.J. Higgins, eds., 1985); "Transcription and Translation" (B.D. Hames & S.J. Higgins, eds., 1984); "Animal Cell Culture" (R.I. Freshney, ed., 1986); "Immobilized Cells and Enzymes" (IRL Press, 1986); B. Perbal, "A Practical Guide to Molecular Cloning" (1984), and Sambrook, et al., "Molecular Cloning: a Laboratory Manual" (1989)). Such techniques are explained fully in the literature, and modification of these techniques within the scope of this invention is within the skill in the art.

The synthetic gene for BoNT serotype B fragment H<sub>C</sub> (see Figure 4A) has been inserted into the yeast expression vector pHIL-D4, and integrated into the chromosome of *Pichia pastoris* strain GS115. The expressed product (see amino acid sequence in Figure 4B) had the expected molecular weight as shown by denaturing polyacrylamide gel electrophoresis (PAGE) and Western blot analysis using antibodies directed against botulinum neurotoxin serotype B. The expressed recombinant BoNTB (H<sub>C</sub>) elicited high antibody titers as judged by the Enzyme Linked Immunosorbent Assay (ELISA) and, more importantly, these circulating serum titers protected mice, guinea pigs, and non-human primates from challenges with active toxin. Industrial scale manufacturing processes (fermentation and

purification) have been completed and a pilot lot has been produced in compliance with cGMP.

The synthetic gene for BoNT serotype C fragment H<sub>C</sub> (see Figure 5A) has been inserted into the yeast expression vector pHIL-D4, and integrated into the chromosome of *Pichia pastoris* strain GS115. The expressed product (see amino acid sequence in Figure 5B) had the expected molecular weight as shown by denaturing polyacrylamide gel electrophoresis (PAGE) and Western blot analysis using antibodies directed against botulinum neurotoxin serotype C. The expressed recombinant BoNTC (H<sub>C</sub>) elicited high antibody titers as judged by the Enzyme Linked Immunosorbent Assay (ELISA) and, more importantly, these circulating serum titers protected mice from challenges with active toxin.

The synthetic gene for BoNT serotype D fragment H<sub>C</sub> (see Figure 6A) has been inserted into the yeast expression vector pHIL-D4, and integrated into the chromosome of *Pichia pastoris* strain GS115. The expressed product (see amino acid sequence in Figure 6B) had the expected molecular weight as shown by denaturing polyacrylamide gel electrophoresis (PAGE) and Western blot analysis using antibodies directed against botulinum neurotoxin serotype D. The expressed recombinant BoNTD (H<sub>C</sub>) elicited high antibody titers as judged by the Enzyme Linked Immunosorbent Assay (ELISA) and, more importantly, these circulating serum titers protected mice from challenges with active toxin.

The synthetic gene for BoNT serotype E fragment H<sub>C</sub> (see Figure 7A) has been inserted into the yeast expression vectors pHILD2, pHILD3, and pPIC9K (see Figures 7B). A modified form of the synthetic gene in which an internal EcoRI site was removed and the gene was enlarged (see Figure 8) was inserted into the yeast vector pHIL-D4, and integrated into the chromosome of *Pichia pastoris* strain GS115. The expressed product (see amino acid sequence in Figure 8) had the expected molecular weight as shown by denaturing polyacrylamide gel electrophoresis (PAGE) and Western blot analysis using antibodies directed against botulinum neurotoxin serotype E. The expressed recombinant BoNTE (H<sub>C</sub>) elicited high antibody titers as judged by the Enzyme Linked Immunosorbent Assay (ELISA) and, more importantly, these circulating serum titers protected mice from challenges with active toxin.

The synthetic gene for BoNT serotype F fragment H<sub>C</sub> (see Figure 9A) has been inserted into the yeast expression vector pHIL-D4, and integrated into the chromosome of *Pichia pastoris* strain GS115. The initial step in the development of the rBoNTF(H<sub>C</sub>) vaccine candidate was to design a gene which could satisfactorily be expressed in a pichia host. A synthetic gene encoding rBoNTF(H<sub>C</sub>) was constructed to lower the inherent AT richness of the native clostridial gene and to remove any potentially rare codons. Clostridial genes having an AT content in excess of 65% or having an average AT content but containing AT-rich tracts usually contain multiple terminators/polyadenylation signals, which can result in premature termination of transcripts when expression is attempted in yeast (Romanos, M.A., et al., (1995), "Expression of Cloned Genes in Yeast," *DNA Cloning 2: Expression Systems*," (Glover D., et al., Eds.), Oxford Univ. Press, London). The synthetic gene in this study required two successive rounds of alterations before the yeast could properly produce full-length antigen. The expressed product (see amino acid sequence in Figure 9B) had the expected molecular weight as shown by denaturing polyacrylamide gel electrophoresis (PAGE) and Western blot analysis using antibodies directed against botulinum neurotoxin serotype F.

A previous study (Hatheway, 1976) demonstrated that the serotype F toxoid antigen needed to be at least partially purified to be efficacious. The same observation was noted with the rBoNTF(H<sub>C</sub>) antigen produced in pichia cells as the crude cell lysate did not protect mice against a BoNTF challenge. The putative receptor-binding domain of BoNTF was purified from yeast and shown to be efficacious in a mouse model. The expressed recombinant BoNTF (H<sub>C</sub>) elicited high antibody titers as judged by the Enzyme Linked Immunosorbent Assay (ELISA) and, more importantly, these circulating serum titers protected mice from challenges with active toxin.

The synthetic gene for BoNT serotype G fragment H<sub>C</sub> (see Figure 10A) has been inserted into the yeast expression vector pHIL-D4, and integrated into the chromosome of *Pichia pastoris* strain GS115. The expressed product (see amino acid sequence in Figure 10B) had the expected molecular weight as shown by denaturing polyacrylamide gel electrophoresis (PAGE) and Western blot analysis

using antibodies directed against botulinum neurotoxin serotype G. The expressed recombinant BoNTG (H<sub>C</sub>) elicited high antibody titers as judged by the Enzyme Linked Immunosorbent Assay (ELISA) and, more importantly, these circulating serum titers protected mice from challenges with active toxin.

5        When purifying a protein for the first time, it is important to generate a viable means for identifying which fractions contain product. If the protein of interest is not an enzyme or does not absorb at a unique wavelength, there are still suitable assays (for example mass spectrometry) for identifying the product. The inventors chose to monitor the purification of rBoNT(H<sub>C</sub>) through immunological  
10        detection by Western blot analysis. However, with various polyclonal antibodies against whole toxin available but without an appropriate positive control, the Western blot results can only be interpreted as ambiguous until a purified sample is sequenced or shown to be protective.

15        There are two major issues of concern when extracting C-fragment, H<sub>N</sub>, and/or heavy chain (H<sub>C</sub>) antigens from pichia cells. The first concern is the solubility of these proteins (i.e., can enough product be extracted into the soluble fraction for further processing?). The second concern deals with the effective removal of polynucleic acids and/or other contaminating materials, which strongly interfere with the necessary chromatography.

20        The zwitterionic detergent, CHAPS, is most notably an effective agent for solubilizing membrane proteins. Membrane proteins exist in a hydrophobic environment, and if removed from that environment, possess strong tendencies to aggregate and ultimately precipitate. CHAPS prevents that aggregation from occurring with membrane bound proteins. The inventors extrapolated this premise  
25        to the clostridial proteins noted above. C-fragments, translocation domains (H<sub>N</sub>), and entire heavy chains are missing their natural partner (the remaining segments of the neurotoxin) and thus, presumably bare exposed hydrophobic regions on their protein surface where the H<sub>C</sub>, H<sub>N</sub>, or heavy chain normally associates with rest of the neurotoxin. These exposed hydrophobic regions are potential nucleation sources  
30        for protein aggregation, because the natural tendency of a protein in an aqueous environment is to bury their hydrophobic surface. When pichia cells are disrupted with CHAPS (on the order of 0.3% W/V) present in the cracking buffer, the amount

of fragment C protein isolated in the soluble fraction has been observed to increase from less than 5% to nearly 80% with serotype C<sub>1</sub>. Dramatic increases in solubility have been noted with C-fragment serotypes A and F as well.

Once a soluble antigen has been produced, the subsequent task is to separate  
5 that antigen from the myriad of pichia host proteins, lipids, and other impurities that exist in the extracted medium. In order for the chemical separations to be feasible by liquid chromatography, it is critical that polynucleic acids be efficiently removed. Nucleotides will either bind to the C-fragment (serotypes A, E, and F due to their elevated pIs) or will bind to the anion-exchange chromatography resin (as is used in  
10 the first purification step of the C<sub>1</sub> process). With either case, the chromatography is rendered futile. The C-fragment product will either fail to bind to the chromatography media or it will elute over an unacceptably large sodium chloride concentration range. Pichia cells possess an abundant amount of DNA. Polyethyleneimine (PEI) is a polycationic agent that readily precipitates nucleotides.  
15 When pichia cell extracts are treated with PEI, the nucleic acids are efficiently precipitated and removed by centrifugation without significant loss of product. More importantly, the chromatographic separation of C-fragments from pichia proteins are dramatically improved.

The soluble portion of the cell lysate may typically be purified in two  
20 conventional chromatographic steps. The ultimate objective of this work is to obtain FDA licensure of rBoNT as a safe and effective vaccine. Even though separations can be accomplished at extremely high resolution with affinity chemistry, there remains an undesirable effect of hapten leaching from the resin. Thus, a preferred separation employs a cation-exchange step followed by hydrophobic interaction  
25 chromatography (HIC). These two steps complement each other as they provide separations based on electrostatic and hydrophobic interactions. The cation-exchange step was particularly efficient in increasing the purity of rBoNTF(H<sub>C</sub>), as the antigen was estimated to be purified greater than 52-fold. The efficiency of purification is primarily attributed to the significant difference in isoelectric points  
30 between most pichia proteins (pIs<7 ) and rBoNT(H<sub>C</sub>) (experimental pI=9.4 for rBoNTF(H<sub>C</sub>) , data not shown) and thus, the pichia proteins were removed in the column flow through. Precipitate that results when the cation-exchange pool is

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treated with ammonium sulfate contains mostly pichia proteins and very little rBoNT product. The HIC step removes most or all of the remaining impurities. The yield of soluble rBoNTF(H<sub>C</sub>) from the total recombinant yeast cell lysate was estimated to be greater than 28% with a purity greater than 98%. Use of similar  
5 purification steps for rBoNTA(H<sub>C</sub>) produced greater than 95% pure material.

A significant amount of rBoNTF(H<sub>C</sub>) product (30-40%) was identified in the insoluble portion of the cell lysate. Also, the antigen was 35% of the total protein present in the pellet; in effect it was more pure than the soluble rBoNTF(H<sub>C</sub>) was after the ion-exchange step. This suggests an alternative process whereby insoluble  
10 rBoNT product produced in yeast may be resolubilized and purified to homogeneity. The resolubilization may be performed by resuspending the pellet in urea and subsequently removing the urea by dialyses in nondenaturing buffer. A single chromatographic step using cation-exchange chemistry may be sufficient to purify the resolubilized antigen, in some cases to greater than 98%. The yield of  
15 resolubilized rBoNTF(H<sub>C</sub>) product from the total cell lysate was estimated to be >19%. The overall bench scale yield of purified soluble and resolubilized rBoNTF(H<sub>C</sub>) was estimated to be greater than 47% or 240 mg/Kg of the cell paste. A similar procedure would be suitable for purification of rBoNTA(H<sub>C</sub>) and other rBoNT fragment peptides from yeast.

20 Analysis of CD spectra of both soluble and resolubilized product revealed the presence of significant  $\beta$ -sheet which is in agreement with that predicted for rBoNTF(H<sub>C</sub>) using an artificial neural network (Lebeda, F.J., et al., (1997), "Predicting Differential Antigen-Antibody Contact Regions Based on Solvent Accessibility," *J. Protein Chem.*, **16**:607-618), and that determined by crystal  
25 structure of BoNT serotype A (Lacy, D.B., et al., (1998), "Crystal Structure of Botulinum Neurotoxin Type A and Implications for Toxicity," *Nat. Struct. Biol.*, **5**:898-902). However, even though CD revealed that the two antigens possessed similar folds, there were subtle differences between the two spectra suggesting that the secondary structures, and hence, tertiary structures were not identical.

### 30 Immunization

The purified soluble and resolubilized antigens appear to be in a folded conformation. However, the bottom line with any potential vaccine is the



demonstration of protection. Are the antigens in a conformation that will elicit the production of neutralizing antibody? To answer this question, mice were inoculated with rBoNTF(H<sub>C</sub>) and subsequently challenged with a high level of rBoNTF toxin. The purified soluble rBoNTF(H<sub>C</sub>) completely protected mice receiving three  
5 inoculations of 0.2 µg from challenge with 1000 mouse i.p. LD<sub>50</sub> of BoNT/F toxin. Analysis of the association of dose with survival indicated that dose was associated with the odds of surviving (odds ratio=2.0, meaning that the odds of survival increase twofold per unit increase in dose with a 95% confidence level from 1.3 to 3.1). The number of inoculations was also associated with survival. Both two  
10 inoculations and three inoculations were associated with increased odds of survival relative to a single inoculation (5.3-fold with a 95% confidence level of 1.2-23 for two inoculations and 22-fold with a 95% confidence level of 4.3 -110 for three inoculations). It is apparent that a single shot at higher doses achieved protection comparable to multiple inoculations at lower doses. Also, three doses of 1 µg of  
15 purified resolubilized rBoNTF(H<sub>C</sub>) completely protected mice from a challenge of 5000 mouse i.p. LD<sub>50</sub> of BoNTF toxin, thus demonstrating that refolded rBoNTF(H<sub>C</sub>) from the insoluble fraction of lysate could also be a prosperous source of antigen.

Individual antibody ELISA titer appears to be an excellent predictor of  
20 mouse survival. If the antibody titer of a mouse was 100 or greater, that mouse was predicted to and did survive a challenge of 1000 mouse i.p. LD<sub>50</sub> of BoNTF toxin. Upon vaccination of mice with 2 or 3 doses of rBoNTA(H<sub>C</sub>) or rBoNTB(H<sub>C</sub>) vaccine delivered on a specific schedule (i.e., parental intramuscular injection at 0,4, and 8 weeks), survival of animals challenged with 100,000 or 1,000,000 million  
25 LD<sub>50</sub> of toxin is very high. Measurement of the antibody levels in these animals via an ELISA shows that the survival rate can be correlated with the measured antibody level. The ELISA is performed by coating a microtiter plate with toxin or fragment C itself, then sera from the vaccinated mice is added at various dilutions (i.e., sera diluted 1/100, 1/400, 1/1600, 1/6400, etc.). Since fragment C is sufficient to elicit  
30 protection in animals, preferably assays for neutralizing antibody titer in sera from animals vaccinated with fragment C are performed using microtiter plates coated with fragment C. Antibody in the sera will bind to the toxin or the fragment C, and the

bound antibody may be detected by a secondary antibody (e.g., anti-mouse IgG) that is coupled to horse-radish peroxidase or alkaline phosphatase. The secondary IgG will bind to the anti-BoNT antibody that was raised to the fragment C vaccine. After washing the microtiter wells, a substrate for the peroxidase or phosphatase enzymes is added to the wells. The substrate will give off a color once the enzyme has cleaved the substrate, and the intensity of the color measured (e.g., at 405 nm. Typically, a reading of 0.2 is used as the base. Thus, if dilution of the sera by 1/1600 gives a reading of 0.15 at 405 nm and a dilution of 1/400 gives a reading of 0.45 at 405 nm, the antibody titer in the sera is characterized as 1/400 dilution (i.e., titer of 400 fold). Obviously, if readings of 0.2 are obtained at higher dilutions, better protection is observed. With rBoNTA(Hc) vaccination, for mice which had ELISA titers of less than 100, only 14.3% survival rate was observed under the conditions of vaccination and challenge. With rBoNTF(Hc), for mice which had ELISA titers of 100 fold, under the condition of vaccination and challenge, 100% of the mice were protected.

It also will be well known to one of ordinary skill in the art that a susceptible host may be immunized using the appropriate peptide vaccine formulated in adjuvant to increase the immune response. Such adjuvants include but are not limited to Freund's (complete and incomplete), mineral gels, such as aluminum hydroxide, surface active substances such as keyhole limpet hemocyanin, lysolecithin, pluronic polyols, polyanions, peptides, BCG (Bacille Calmette-Guerin), oil emulsions and dinotrophenols. Immunization can be carried out with additional various presentation and cross-linking permutations. By way of example and not of limitation, such permutations include rBoNT peptides cross-linked to KLH as a carrier, any rBoNT peptide cross-linked to any other rBoNT protein as carrier, rBoNT peptides cross-linked to themselves, and these combinations presented by the various adjuvants listed above. It will become evident that such permutations are available in regard to other peptides and self-assembled peptides disclosed throughout this specification.

It will also be known to one of ordinary skill in the art that use of the term "susceptible host" includes any such mammalian host susceptible to intoxication by BoNT. It will be further evident that any such susceptible host is a candidate for

treatment to promote protection from BoNT utilizing the peptide vaccines and associated methods described in this specification.

## EXAMPLES

5 In order to facilitate a more complete understanding of the invention, a number of Examples are provided below. However, the scope of the invention is not limited to specific embodiments disclosed in these Examples, which are for purposes of illustration only.

### 10 Example 1. Synthesis and cloning of a synthetic gene encoding rBoNTF(H<sub>C</sub>)

A synthetic gene encoding a putative fragment C region of botulinum neurotoxin serotype F was designed and constructed for expression in *Escherichia coli* (Holley *et al.*, submitted to Vaccine). The recombinant BoNTF(H<sub>C</sub>)<sub>1</sub> gene was expressed in *E. coli* as a fusion protein with maltose-binding protein (MBP) with  
15 yields of 1 mg/L culture (See Figure 18).

The same gene was used for expression studies in the yeast, *P. pastoris*. This particular host was chosen because it could produce high levels of recombinant proteins (Cregg, J.M., et al., (1993), "Recent Advances in the Expression of Foreign Genes in *Pichia pastoris*," *Bio/Technology*, **11**:905-909; Romanos, M.A., et al.,  
20 (1992), "Foreign Gene Expression in Yeast: A Review," *Yeast*, **8**:423-488; Sreekrishna, K., et al., (1988), "High Level Expression of Heterologous Proteins in Methylophilic Yeast *Pichia pastoris*," *J. Bas. Microbiol.*, **28**:265-278) and because it lacked endotoxins which would facilitate product development. Intracellular expression of the antigen was used to avoid potential glycosylation of the  
25 recombinant protein. The rBoNTF(H<sub>C</sub>)<sub>1</sub> gene was modified at its 3' end for insertion into the unique EcoR I site of the yeast vector, pHILD4. The recombinant construct containing the rBoNTF(H<sub>C</sub>)<sub>1</sub> gene was subsequently linearized with Sac I and the cassette integrated into the chromosomal alcohol oxidase (AOX 1) of *Pichia pastoris* strain GS115 (Clare, J.J., et al., (1991), "High-Level Expression of Tetanus  
30 Toxin Fragment C in *Pichia pastoris* Strains Containing Multiple Tandem Integrations of the Gene," *Bio/Technology*, **9**:455-460). Yeast transformants expressing the selectable markers histidine dehydrogenase (Cregg, J.M., et al.,

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(1985), "*Pichia pastoris* as a Host System for Transformations," *Mol. Cell. Biol.*, **5**:3376-3385) and aminoglycoside phosphotransferase 3' (I) (Scorer, C.A., et al., (1994), "Rapid Selection Using G418 of High Copy Number Transformants of *Pichia pastoris* for High-Level Foreign Gene Expression," *Bio/Technology*, **12**:181-184) were isolated. These isolates were further characterized for their ability to express rBoNTF(H<sub>C</sub>) after induction with methanol. Although the various transformants generated were able to express the selectable markers, no expression of rBoNTF(H<sub>C</sub>) as judged by SDS/PAGE and blot analysis was observed in these isolates (data not shown).

10           **SDS/PAGE, Western blot, and protein assays**

          Total protein concentrations were determined by using the Pierce BCA™ (bicinchoninic acid) protein assay kit with BSA as a standard. The purity of the rBoNTF(H<sub>C</sub>) product was assessed by SDS/PAGE with Novex (San Diego, CA, U.S.A.) gel electrophoresis supplies, reagents, protocols, and National Institutes of Health (NIH) imaging software as previously described (Byrne, M.P., et al., (1998), "Purification, Potency, and Efficacy of the Botulinum Neurotoxin Type A Binding Domain from *Pichia pastoris* as a Recombinant Vaccine Candidate," *Infect. Immun.*, **66**:4817-4822). Western blot assays were used to identify FPLC fractions containing rBoNTF(H<sub>C</sub>) as previously described (Byrne, 1998) with the following changes. The primary antibody used was a polyclonal protein G sepharose-purified horse anti-BoNTF antibody incubated at 1 µg/ml for 3 h. The secondary antibody used was a horseradish peroxidase-labeled affinity-purified goat anti-horse IgG (Kirkegaard & Perry Laboratories, Gaithersburg, MD, U.S.A.) assayed at 1 µg/ml for 2 h.

25           **Example 2. Synthesis and cloning of a synthetic gene encoding rBoNTF(H<sub>C</sub>)**

          A second synthetic gene, rBoNTF(H<sub>C</sub>)<sub>2</sub>, was subsequently designed to facilitate expression in *P. pastoris*. Redesigning the gene was intended to lower specific regions of the rBoNTF(H<sub>C</sub>)<sub>1</sub> gene in which spikes of AT-rich tracts still remained. Previous work had shown that rare codons (Makoff, A.J., et al., (1989), "Expression of Tetanus Toxin Fragment C in *E. coli*: High Level Expression by Removing Rare Condons," *Nucleic Acids Res.*, **17**:10191-10201) and/or highly enriched AT base compositions (Romanos, M.A., et al., (1991), "Expression of

Tetanus Toxin Fragment C in Yeast: Gene Synthesis is Required to Eliminate Fortuitous Polyadenylation Sites in AT-rich DNA," *Nucleic Acids Res.*, **19**:1461-1467) in clostridial DNA were incompatible with optimum expression of clostridial genes in *E. coli* and yeast. A second synthetic gene encoding the rBoNTF(H<sub>C</sub>) fragment was designed and constructed using *P. pastoris* codon usage (Sreekrishna, K., (1993), "Strategies for Optimizing Protein Expression and Secretion in the Methylophilic Yeast *Pichia pastoris*," *Industrial Microorganisms: Basic and Applied Molecular Genetics*, (Baltz, R. H., et al, Eds.), pp. 119-126, Am. Soc. Microbiol., Washington, DC). Briefly, complimentary oligonucleotides encoding the amino terminal region of the F(H<sub>C</sub>) (423 nucleotides flanked with *EcoRI* and *PstI* sites), the central region of the F(H<sub>C</sub>) (606 nucleotides flanked by *PstI* and *SalI* sites) and the carboxy-terminal region of F(H<sub>C</sub>) (336 nucleotides flanked by *SalI* and *EcoRI* sites) were annealed and cloned into pUC or PCR zero-blunt plasmid vectors. The AT base composition in the native clostridial F(H<sub>C</sub>) DNA averaged 76% while rBoNTF(H<sub>C</sub>)<sub>1</sub> averaged 58% and rBoNTF(H<sub>C</sub>)<sub>2</sub>, 53% (Figure 19). The synthetic gene sequence of rBoNTF(H<sub>C</sub>)<sub>2</sub> and the 432 amino acids it encoded for is shown in Figure 9. After nucleotide sequencing, the cloned fragments were excised by the appropriate restriction endonucleases, separated by agarose gel electrophoresis, and purified. The isolated DNA fragments were ligated simultaneously into *EcoR* I digested and dephosphorylated plasmid pHIL4. The vector harboring the rBoNTF(H<sub>C</sub>)<sub>2</sub> gene was integrated into the chromosomal *AOX1* locus of *P. pastoris* as described above. Transformants expressing selectable markers (histidine dehydrogenase and aminoglycoside phosphotransferase 3' (I)) were isolated and tested for their ability to express rBoNTF(H<sub>C</sub>). Unlike the rBoNTF(H<sub>C</sub>)<sub>1</sub> gene, rBoNTF(H<sub>C</sub>)<sub>2</sub> was expressed after induction with methanol and yielded the expected molecular weight of approximately 50000 daltons as judged by SDS/PAGE and Western blot analysis (Figure 20). The deduced molecular mass of the encoded polypeptide was 50,250 daltons.

### Example 3. Expression and Cell Disruption of rBoNTF(H<sub>C</sub>) in *P. pastoris*

Large-scale fermentation conditions and optimal intracellular expression of rBoNTF(H<sub>C</sub>) were determined for the yeast strain *P. pastoris*.

### Protein expression

A stock seed culture of *P. pastoris* was grown in shake-flasks containing 0.5 L of YNB medium (13.4 g/L yeast nitrogen base without amino acids, 20 g/L glycerol, 0.4 mg/L biotin, in 100 mM sodium phosphate, pH 6.0). Cultures were grown at 30°C until an  $A_{600}$  of 20 absorbance units was achieved, and then used to inoculate a 5-L BioFlo 3000 fermentor (New Brunswick Scientific, Edison, NJ, U.S.A.) containing 2.5 L basal-salt medium plus PTM<sub>4</sub> trace mineral salts and 4% glycerol. Dissolved oxygen was maintained at 40% and the pH was maintained at 5.0 with 30% ammonium hydroxide. After the initial glycerol was consumed, 50% (w/v) glycerol was added at a rate of 20 g/L/h for 1 h then decreased linearly to 0 g/L/h over 3 h. The medium was enriched with 1.5 g methanol/L of medium. Methanol feed was started at 4 g/L/h and linearly increased to 9 g/L/h over 10 h. The methanol feed rate was adjusted by using the dissolved oxygen-spike method (Chiruvolu, V., et al., (1997), "Recombinant Protein Expression in an Alcohol Oxidase-Defective Strain of *Pichia pastoris* in Feed-Batch Fermentations, *Enzyme Microbiol. Technol.*, **21**:277-283). After 10 h of methanol induction, the cells were harvested by centrifugation at 6000 g for 10 min at 4°C with a Beckman JA-10 rotor (Beckman Instruments, Palo Alto, CA, U.S.A.) and then stored at -20°C.

### Protein expression

A stock seed culture of *P. pastoris* was grown in shake-flasks containing 0.5 L of YNB medium (13.4 g/L yeast nitrogen base without amino acids, 20 g/L glycerol, 0.4 mg/L biotin, in 100 mM sodium phosphate, pH 6.0). Cultures were grown at 30°C until an  $A_{600}$  of 20 absorbance units was achieved, and then used to inoculate a 5-L BioFlo 3000 fermentor (New Brunswick Scientific, Edison, NJ, U.S.A.) containing 2.5 L basal-salt medium plus PTM<sub>4</sub> trace mineral salts and 4% glycerol. Dissolved oxygen was maintained at 40% and the pH was maintained at 5.0 with 30% ammonium hydroxide. After the initial glycerol was consumed, 50% (w/v) glycerol was added at a rate of 20 g/L/h for 1 h then decreased linearly to 0 g/L/h over 3 h. The medium was enriched with 1.5 g methanol/L of medium. Methanol feed was started at 4 g/L/h and linearly increased to 9 g/L/h over 10 h. The methanol feed rate was adjusted by using the dissolved oxygen-spike method (Chiruvolu, 1997). After 10 h of methanol induction, the cells were harvested by

centrifugation at 6000 x g for 10 min at 4°C with a Beckman JA-10 rotor (Beckman Instruments, Palo Alto, CA, U.S.A.) and then stored at -20°C.

### Cell disruption and sample preparation

Eleven g of frozen cell paste was resuspended in 100 ml of 50 mM Na<sub>2</sub>HPO<sub>4</sub>/2 mM Na<sub>2</sub>EDTA/1 mM PMSF, pH 6.8 at 4°C. The suspended cells were disrupted by three successive passes through a microfluidizer device (model 110Y, Microfluidics Corp., Newton, MA, U.S.A.) at 21000 psi. The temperature of the disruptate was kept below 10°C throughout the process by cooling the exit line and collection flask with ice. The cells were judged to be greater than 95% disrupted as determined by microscopy. In comparison, 8-10 passes through a Gaulin homogenizer were required to efficiently disrupt the cells in past protocols. SDS-PAGE and Western blot analysis of cell lysate showed that expressed rBoNTF(H<sub>C</sub>) represented <0.5% of the total protein. The resulting cell lysate volume was 105 ml with a protein concentration of 11 mg/ml. Cellular debris and insoluble proteins were removed by centrifugation at 15000 g for 15 min at 4°C with a Sorval SS-34 rotor (Sorval Instruments, Newtown, CT, U.S.A.). The resulting extract was noticeably turbid due to the presence of lipids and significant quantities of nucleic acids. As rBoNTF(H<sub>C</sub>) possessed a calculated isoelectric point of 9.1 and presumably interacted strongly with DNA, DNase was added to the cell extract in order to digest the polynucleotides and facilitate purification. To remove the polynucleotides, the extract was treated with DNase (100 units/ml, Aldrich) and ZnCl<sub>2</sub> (2 mM, Aldrich) at room temperature for 30 min and then dialyzed extensively with 10 kDa molecular weight cut off (MWCO) Slide-A-Lyzer dialysis cassettes (Pierce) in 50 mM Na<sub>2</sub>HPO<sub>4</sub>/2 mM Na<sub>2</sub>EDTA/1 mM PMSF, pH 6.8 at 4°C. A precipitate developed during dialysis that was separated by centrifugation at 15000 g for 15 min at 4°C with a Sorval SS-34 rotor. The clarified extract contained 7.8 mg/ml of total protein and was used as starting material for the FPLC purification of soluble rBoNTF(H<sub>C</sub>) while the pellet was used as starting material for the resolubilized rBoNTF(H<sub>C</sub>) purification.

**Example 4. Conventional Purification of rBoNTF(H<sub>C</sub>) from *P. pastoris***

The rBoNTF(H<sub>C</sub>) protein was purified to homogeneity using an FPLC system and two chromatographic steps. First, the material was subjected to cation exchange chromatography (Figure 21A).

5      **FPLC purification of soluble rBoNTF(H<sub>C</sub>)**

Soluble rBoNTF(H<sub>C</sub>) was purified by using a Pharmacia model 500 FPLC system (Pharmacia, Uppsala, Sweden) with programmed elution and A<sub>280</sub> monitoring. The starting material was loaded onto a Pharmacia HR 10/10 Mono S cation-exchange column equilibrated with 50 mM Na<sub>2</sub>HPO<sub>4</sub>/2 mM Na<sub>2</sub>EDTA/1 mM  
10 PMSF, pH 6.8 (buffer A) at a flow rate of 2 ml/min (150 cm/h). The column was washed with 16 ml (2 bed volumes) of buffer A. Flow through and wash were collected separately and stored for subsequent analysis. Protein was eluted from the column with a linear gradient from 0 to 300 mM NaCl over 80 ml (10 bed volumes), then a linear gradient from 300 to 1000 mM NaCl over 20 ml (2.5 bed volumes), and  
15 then an isocratic gradient at 1000 mM over 10 ml (1.25 bed volumes). Four-ml fractions were collected throughout the linear and isocratic gradients. This step was highly efficient as most pichia proteins possess isoelectric points between pH 5 and 7 and, therefore, pass through the column without binding. Fractions eluting between 230 and 260 mM NaCl were positive for rBoNTF(H<sub>C</sub>) by Western blot  
20 analysis and were pooled. The pooled fractions were adjusted to 1.5 M ammonium sulfate by the slow addition of 2 M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>/50 mM Na<sub>2</sub>HPO<sub>4</sub>/2 mM Na<sub>2</sub>EDTA/25 mM NaCl, pH 7.5 with stir bar agitation. A protein precipitate formed which consisted primarily of yeast proteins with a small amount of rBoNTF(H<sub>C</sub>) product (approximately 10%). The precipitate was removed by centrifugation at  
25 6000 g for 10 min at 4°C with a Sorval SS-34 rotor. Fortunately, when the pool of Mono S column fractions was diluted with ammonium sulfate, most of the rBoNTF(H<sub>C</sub>) product remained in solution (approximately 90%) while significant quantities of pichia proteins salted out. The first step enriched the desired product from <0.5 to 26% of the total protein (Table 2).

30      HIC was used as a second chromatographic step (Figure 21B) and separated proteins based on their differences in surface hydrophobicity. It was determined that neopentyl chemistry provided the appropriate hydrophobic interaction with

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rBoNTF(H<sub>C</sub>). The supernatant was loaded onto a Pharmacia alkyl superose 10/10 hydrophobic interaction chromatography (HIC) column equilibrated with 1.5 M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>/50 mM Na<sub>2</sub>HPO<sub>4</sub>/2 mM Na<sub>2</sub>EDTA/25 mM NaCl, pH 7.5 (buffer B) at a flow rate of 1 ml/min (75 cm/h). The column was washed with 8 ml (1 bed volume) of buffer B. Protein was eluted from the column with a linear gradient of decreasing (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> from 1.5 to 0 M over 60 ml (7.5 bed volumes). The rBoNTF(H<sub>C</sub>) eluted from the HIC column at 0.92 M ammonium sulfate in a volume of 3 ml with a protein concentration of 0.52 mg/ml. Fractions positive by Western blot analysis and which only showed a single band by SDS/PAGE were pooled and dialyzed extensively in 50 mM Na<sub>2</sub>HPO<sub>4</sub>/2 mM Na<sub>2</sub>EDTA, pH 6.8.

The recovery of purified product from cell extract was estimated to be greater than 42%, with a yield of 140 mg/kg of cell paste (Table 2). The resulting rBoNTF(H<sub>C</sub>) was judged to be greater than 98% pure as only a single band was detected by SDS-PAGE (Figure 20) even when moderately (4 μg) overloaded. Capillary isoelectric focusing showed the antigen possessed an isoelectric point of 9.4 (data not shown), which is in reasonable agreement with the calculated pI of 9.1.

TABLE 2. Purification of soluble rBoNTF(H<sub>C</sub>)

Total protein concentration was determined by Pierce BCA™ assay. rBoNTF(H<sub>C</sub>) was identified by Western blot analysis and purity was estimated by analysis of individual lanes of SDS/PAGE by pixel densitometry using NIH imaging software.

Step	Concentration (mg/ml)	Protein (mg)	rBoNTF(H <sub>C</sub> ) (mg)	Purity (%)	Fold Purification	Recovery (%)
Lysate	11	1100	5.6	<0.5	--	3.8
Dialzed extract	7.8	740	3.7	<0.5	--	66
Mono S	1.2	9.6	2.5	26	>52	45
Alyl superose	0.52	1.6	1.6	100	3.8	29

#### CD of purified soluble and resolubilized rBoNTF(H<sub>C</sub>)

Purified soluble and resolubilized rBoNTF(H<sub>C</sub>) were subjected to CD spectroscopy in a Jasco 600 spectropolarimeter (Japan Spectroscopy company, Tokyo, Japan). Experiments were performed at a concentration of 30 μg/ml (0.62 μM) in a 1 cm path length cell in 10 mM Na<sub>2</sub>HPO<sub>4</sub>, pH 7.0. Spectra were obtained

as an average of four accumulations, scanned from 260-200 nm, at a scan rate of 10 nm/min, with a 2 sec response, and a 1 nm band width. The temperature was maintained at 20°C with a Peltier thermocontrol device.

Analysis of the far-UV circular dichroism spectrum (Figure 22) of the purified antigen showed a positive peak at 233 nm and a minimum at 214 nm. This suggests the molecule is in a folded conformation and possesses considerable  $\beta$ -sheet.

#### **Example 5. Purification of Resolubilized rBoNTF(H<sub>C</sub>)**

Western blot analysis revealed that approximately 30-40% of the total expressed rBoNTF(H<sub>C</sub>) was present in the insoluble pellet after cell lysis. To investigate whether this insoluble protein could be recovered, the pellet was extracted in the denaturant urea and then dialyzed in nondenaturing buffer.

#### **FPLC purification of resolubilized rBoNTF(H<sub>C</sub>)**

The cell lysate pellet was resuspended into 20 ml of 3 M urea/50 mM Na<sub>2</sub>HPO<sub>4</sub>, pH 7.0 and extracted 15 h at 4°C on a Labquake rotator. The cellular components not solubilized by the denaturing buffer were removed by centrifugation with a Sorval SS-34 rotor at 15000 g for 10 min at 4°C. The supernatant was dialyzed extensively using 10 kDa MWCO Pierce Slide-A-Lyzer dialysis cassettes in buffer A. A slight precipitate formed during the dialysis which was removed by centrifugation as described above. Western blot analysis showed that rBoNTF(H<sub>C</sub>) was present only in the supernatant, which was estimated to be about 35% pure by SDS/PAGE. The supernatant was loaded onto a Pharmacia HR 10/10 Mono S cation-exchange column and separated by the same conditions as above. Fractions containing only a single positive rBoNTF(H<sub>C</sub>) band SDS/PAGE and Western blot analysis were pooled and dialyzed in 50 mM Na<sub>2</sub>HPO<sub>4</sub>/2 mM Na<sub>2</sub>EDTA, pH 6.8 in 10 kDa MWCO dialysis cassettes. The final resolubilized rBoNTF(H<sub>C</sub>) product was judged to be greater than 98% pure as determined by SDS/PAGE.

After a single cation exchange chromatography separation step, the rBoNTF(H<sub>C</sub>) was greater than 98% pure as judged by SDS-PAGE. The total yield of purified resolubilized rBoNTF(H<sub>C</sub>) was 100 mg/kg of cell paste. The conformation of purified resolubilized antigen showed significant  $\beta$ -sheet as determined by CD spectral analysis (Figure 22). However, the overall fold appeared

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slightly different than that shown by rBoNTF(H<sub>C</sub>) purified from the cell lysate supernatant. The primary difference was the lack of a positive peak at 233 nm, indicating differences in  $\beta$ -sheet content.

#### **Example 6. Mouse Immunogenicity and Efficacy Studies**

5 To assess the immunogenicity of the recombinant rBoNTF(H<sub>C</sub>), mice were inoculated with either one, two, or three doses of purified rBoNTF(H<sub>C</sub>) from the soluble fraction of lysate at doses ranging from 0.008 to 5  $\mu$ g per mouse.

#### **Mouse inoculations and BoNTF toxin challenge**

10 Mice, Crl:CD-1, ICR mice (Charles River, NC, U.S.A.) weighing 16-22 g on receipt, were injected intramuscularly (i.m.) with purified rBoNTF(H<sub>C</sub>). Mice were challenged intraperitoneally (i.p.) 21 days after their last rBoNTF(H<sub>C</sub>) injection with BoNTF toxin complex (Langeland strain) diluted in .02% (w/v) gelatin/0.4% (w/v) Na<sub>2</sub>HPO<sub>4</sub>, pH 6.2, in 100  $\mu$ l total volume per mouse. Groups of five naïve mice were also used as toxin controls. Mice were observed daily and deaths were recorded five  
15 days post challenge. All animal manipulations were in accordance with applicable regulations in AAALAC-accredited facilities.

The efficacy of the purified soluble rBoNTF(H<sub>C</sub>) was determined by inoculating groups of five female mice with one, two, or three doses of 0.008, 0.04, 0.2, 1.0, or 5.0  $\mu$ g rBoNTF(H<sub>C</sub>) (diluted in 100  $\mu$ l of 0.2% (v/v) Alhydrogel  
20 (Superfos Biosector, Kvistgaard, Denmark) in 0.9% (w/v) saline) per mouse at 14 day intervals. Two days before challenge, mice were bled retroorbitally and serum was collected for ELISA testing. Mice were challenged with 1000 mouse i.p. LD<sub>50</sub> of BoNTF toxin complex.

All of the mice, including five naïve controls, were challenged with 1000  
25 mouse ip LD<sub>50</sub> of BoNTF toxin. The controls all died within 2-4 h. A dose response was observed from groups of mice receiving different numbers of inoculations (Table 3). A single inoculation of 5  $\mu$ g protected four of five mice, while a dose of 0.2  $\mu$ g or below protected one or no mice. Two and three inoculations protected four of five and five of five mice at doses of 0.2 and 0.04  $\mu$ g,  
30 respectively. At all dose levels studied, the number of surviving mice increases with the number of inoculations.

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Serum antibody titers for each individual mouse were determined by ELISA, followed by calculation of the geometric mean titers for each group in the study.

### Mouse serum ELISA

Individual mouse serum ELISAs were performed as previously described (Byrne, 1998) except for the following differences. Botulinum neurotoxin serotype F (Langeland strain, Food Research Institute, University of Wisconsin, Madison, WI, U.S.A.) was used as the coating antigen and the positive control for each assay was a mouse IgG monoclonal antibody, 7F8.G2.H3 (Brown, D.R., et al., (1997), "Identification and Characterization of a Neutralizing Monoclonal Antibody Against Botulinum Neurotoxin, Serotype F, Following Vaccination with Active Toxin," *Hybridoma*, **16**:447-456).

**TABLE 3. Survival, antibody group ELISA titers, and serum neutralization titers of mice after inoculation with purified soluble rBoNTF(H<sub>0</sub>)**

Mice were challenged with 1000 i.p. LD<sub>50</sub> BoNTF toxin 21 days after last inoculation. Antibody ELISA titers were measured as the reciprocal of the highest dilution having an OD<sub>405</sub> greater than 0.2 AU after correcting for background. Geometric mean ELISA titers were determined by taking the geometric mean of the logarithm of the individual titers. Standard deviations of the geometric means are also reported. If the ELISA titer was determined to be below the detection limit of the assay (<100), the ELISA titer was arbitrarily assigned a value of 25. A geometric mean titer value of 1.4 means that all ELISA titers within that group were below the detection limit.

Vaccination dose (µg)	Survival (alive/5 tested)			Geometric mean ELISA titers		
	1X	2X	3X	1X	2X	3X
0.008	0*	0	2	1.4	1.4	1.6±0.3
0.04	0	1	4	1.4	1.5±0.3	2.4±0.8
0.2	1	4	5	1.4	2.1±0.9	2.9±1.3
1.0	2	5	5	1.4	2.8±0.3	4.3±0.3
5.0	4	4	5	1.6±0.3	2.8±0.9	4.1±0.5

\* Only four mice were tested within this group

### Statistical analysis

The logistic regression model was used to test associations of geometric mean ELISA titers and individual titers with survival by using SAS, version 6.10.

5 Geometric mean titers correlated well with protection (Table 3). The three groups with no survivors had geometric means titers below the detection limit of the assay (1.4). Similarly, the four groups that showed complete protection had geometric means titers of 2.8 or greater. Individual mouse antibody titers correlated extremely well with protection (Table 4). Only 7 out of 38 mice survived whose titers were  
10 below 100. On the other hand, 34 out of 34 survived whose titers were 100 or greater. One mouse in the study could be classified as a "nonresponder." The mouse, receiving two injections at the highest dose level, had an antibody titer below the detection limit and did not survive the BoNTF challenge. The rest of the mice in that particular group had titers of 1600 or greater.

15

#### **TABLE 4. Correlation of individual antibody ELISA titer with protection after inoculation with purified soluble rBoNTF(H<sub>C</sub>)**

Serum was bled from each mouse individually. Titer is reciprocal of the highest dilution having an OD<sub>405</sub> greater than 0.2 AU after correcting for background. Mice  
20 were challenged with 1000 i.p. LD<sub>50</sub> BoNTF toxin 21 days after last inoculation.

Individual ELISA titer	Survival (alive/total)*	% survival
<100	7/38	18.4
100	7/7	100
400	4/4	100
1600	11/11	100
6400	3/3	100
25600	9/9	100

\* The individual antibody titers from three mice were not measured. Two mice did not offer enough serum and one mouse was not challenged.

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The resolubilized antigen was also evaluated for immunogenicity and protective efficacy by its ability to protect mice from a BoNTF toxin challenge.

5 Groups of 10 male mice each received three inoculations of either 1  $\mu$ g or 5  $\mu$ g of rBoNTF(H<sub>C</sub>) (diluted in 100  $\mu$ l 0.2% (v/v) Alhydrogel in 0.9% (w/v) saline) per mouse at 14 day intervals. Two days before challenge, mice were bled retroorbitally and serum was collected for ELISA testing. Mice inoculated with 1  $\mu$ g doses were challenged with 5000 mouse ip LD<sub>50</sub> of BoNTF toxin. Ten of ten mice survived the

10 challenge. Because 100% protection was observed with the group inoculated with 1  $\mu$ g doses, the group that received three doses of 5  $\mu$ g were subjected to a challenged level two orders of magnitude greater in order to test the limits of the antigen. Therefore, the 5  $\mu$ g dose group was challenged with 500,000 mouse ip LD<sub>50</sub> of BoNTF toxin. None of the mice survived the challenge; however, a significant

15 delay in time to death was observed (24-48 h). All the control mice succumbed within 2-4 h after challenge.

**Example 7. Synthesis and cloning of a synthetic gene encoding rBoNTA (H<sub>C</sub>)**

The preparation of genetically engineered proteins to provide

20 protection from the toxins produced by *Clostridium botulinum* was accomplished in *E. coli*.

Restriction endonucleases and DNA modifying enzymes were obtained from GIBCO BRL (Gaithersburg, Maryland). Polymerase chain reaction (PCR) reagents were purchased from Perkin-Elmer Cetus (Norwalk, CT). SDS

25 PAGE precast gels and running buffers were acquired from Amersham (Arlington Heights, Illinois). All oligonucleotides were synthesized by Macromolecular Resources (Ft. Collins, Colorado). ELISA reagents were obtained in house or from Sigma (St. Louis, Missouri) or Kirkegard and Perry Laboratories (Gaithersburg, Maryland).

30 The *Escherichia coli* host was K12DH5a, purchased as competent cells from GIBCO BRL. Expression vectors pMAL from New England Biolabs (Beverly, Massachusetts) and pKK233-2 from Pharmacia LKB (Piscataway, New

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Jersey) were used according to the manufacturers' standard protocols. The DNA clone coding of the H<sub>C</sub> domain of *c. botulinum* toxin serotype A was pCBA3, kindly provided by Nigel Minton.

Oligonucleotide primers incorporating appropriate terminal  
5 restriction enzyme sites were used to PCR amplify the H<sub>C</sub> region of the *C.*  
*botulinum* clone pCBA3. Gel-purified insert DNA and vector DNA were cleaved  
with the appropriate restriction enzymes, purified on low melting point agarose, and  
ligated overnight at room temperature. Competent DH5a host cells were  
transformed according to suppliers recommendations and plated on LB plates with  
10 100 ug/ml ampicillin. Protein electrophoresis was run on precast 11-20% SDS  
PAGE at the manufacturer's recommended parameters. ELISA plates were  
incubated with capture antibody (horse anti-botulinum A polyclonal serum)  
overnight, then blocked with skim milk prior to application of various dilutions of  
test material, signal antibody (rabbit antitoxin A polyclonal serum), signal HRP  
15 conjugated anti-(rabbit IgG) and ABTS substrate solution. Plates were read on an  
automated reader at 405 nm.

The sequence of the C fragment of the A chain was deduced as:

RYESNHLIDLSRYASKINIGSKVNFDPIDKNQIQLFNLESSKIEVILKNA  
IVYNSMYENFSTSFWIRIPKYFNSISLNNEYTIINCMENNSGWKVSLNYGEII  
20 WTLQDTQEIKQRVVFYKYSQMINISDYINRWIFVTITNNRLNNSKIYINGRLID  
QKPISNLGNIHASNNIMFKLDGCRDTHRYIWIFYFNLFDKELNEKEIKDLYDN  
QNSNGILKDFWGDYLYQYDKPYMLNLYDPNKYVDVNNVGIRGYMYLKGP  
RGSVMTTNIYLNSSLYRGTKFIIKKYASGNKDNIVRNNDRVYINVVKNKE  
YRLATNASQAGVEKILSALEIPDVGNLSQVVVMKSKNDQGITNKCKMNLQ  
25 DNNGNDIGFIGFHQFNNAKLVASNWNRYQIERSSRTLGCSEFIPVDDGWG  
ERPL

The C fragment protein sequence was reverse translated using *E. coli* optimal  
codon usage. The gene was then altered in many places to insert restriction sites,  
start codon, stop codon. Other changes were also effected to make the molecule  
30 more appropriate for use in the vector. Throughout, the fidelity of the protein  
sequence generated therefrom was maintained.

The sequence for the synthetic gene is found below:

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CTCGAGCCATGGCTCGTCTGCTGTCTACCTTCACTGAATACATCAA  
GAACATCATCAATACCTCCATCCTGAACCTGCGCTACGAATCCAATCACC  
TGATCGACCTGTCTCGCTACGCTTCCAAAATCAACATCGGTTCTAACTT  
AACTTCGATCCGATCGACAAGAATCAGATCCAGCTGTTCAATCTGGAAT  
5 CTTCCAAAATCGAAGTTATCCTGAAGAATGCTATCGTATACAACCTCTATG  
TACGAAAACCTTCTCCACCTCCTTCTGGATCCGTATCCCAAATACTTCAAC  
TCCATCTCTCTGAACAATGAATACACCATCATCAACTGCATGGAAAACA  
ATTCTGGTTGGAAAGTATCTCTGAACTACGGTGAAATCATCTGGACTCTG  
CAGGACACTCAGGAAATCAAACAGCGTGTTGTATTCAAATACTCTCAGA  
10 TGATCAACATCTCTGACTACATCAATCGCTGGATCTTCGTTACCATCACC  
AACAAATCGTCTGAATAACTCCAAAATCTACATCAACGCCGTCTGATCGA  
CCAGAAACCGATCTCCAATCTGGGTAACATCCACGTTCTAATAACATCAT  
GTTCAAACCTGGACGGTTGTCGTGACACTCACCGCTACATCTGGATCAAAT  
ACTTCAATCTGTTGACAAAAGAACTGAACGAAAAAGAAATCAAAGACCT  
15 GTACGACAACCGTCCAATTCTGGTATCCTGAAAGACTTCTGGGGTGACT  
ACCTGCAGTACGACAAACCGTACTACATGCTGAATCTGTACGATCCGAA  
CAAATACGTTGACGTCAACAATGTAGGTATCCGCGGTTACATGTACCTG  
AAAGGTCCGCGTGGTTCTGTTATGACTACCAACATCTACCTGAACTCTTC  
CCTGTACCGTGGTACCAAATTCATCATCAAGAAATACGCGTCTGGTAAC  
20 AAGGACAATATDCGCAACAATGATCGTGTATACATCAATGTTGTAGTTA  
AGAACAAAGAATACCGTCTGGCTACCAATGCTTCTCAGGCTGGTGTAGA  
AAAGATCTTGTCTGCTCTGGAAATCCCGGACGTTGGTAATCTGTCTCAGG  
TAGTTGTAATGAAATCCAAGAACGACCAGGGTATCACTAACAAATGCAA  
AATGAATCTGCAGGACAACAATGGTAACGATATCGGTTTCATCGGTTTCC  
25 ACCAGTTCAACAATATCGCTAAACTGGTTGCTTCCAACCTGGTACAATCGT  
CAGATCGAACGTTCTCTCGCACTCTGGGTTGCTCTTGGGAGTTCATCCC  
GGTTGATGACGGTTGGGGTGAACGTCCGCTGTAACCCGGGAAAGCTT

This gene has been synthesized using a large number of oligomers of approximately 60-65 bases corresponding to the sequences of the + and - strands.

30 The oligomers had overlaps of 7 bases. The oligomers were allowed to anneal and were ligated to form 5 subunits of 250-300 base pairs each. Each subunit had been designed to have restriction sites at their termini which allowed them to be



assembled in the right order to form the complete gene. On confirmation there was shown that the correct gene had 7 deletion errors. These errors were repaired *using in vitro* mutagenesis and the repair sites sequenced to confirm.

**Example 8. Synthesis and cloning of a synthetic gene encoding**  
5 **rBoNTB (H<sub>C</sub>)**

The C fragment for botulism toxin serotype B of Whelan was studied and the portion of the protein having the sequence

FNKYNSEILNNIILNRLRYKDNNLIDLSGYGAKVEVYDGVELNDKNQF  
KLTSSANSKIRVTQNQNIIFNSVFLDFSVSFWIRIPKYKNDGIQNYIHNEYTIIN  
10 CMKNNSGWKISIRGNRITWTLIDINGKTKSVFFEYNIREDISEYINRWFFVTIT  
NNLNNAKIYINGKLESNTDIKDIREVIANGEIIFKLDGDIDRTQFIWMKYFSIF  
NTELSQSNIERYKIQSYSEYLKDFWGNPLMYNKEYYMFNAGNKNSYIKLK  
KDSPVGEILTRSKYNQNSKYINYRDLYIGEKFIIRKSNSSQSINDDIVRKEDI  
YLDFF-NLNQEQRVYTYKKFKKEEEKLFLAPISDSDEFYNTIQIKEYDEQPTY  
15 SCQLLFKKDEESTDEIGLIGIHRFYESGIVFEEYKDYFCISKWYLKEVKRKPY  
NLKLGCNWQFIPKDEGWTE

was defined as the C fragment.

The synthetic gene for expression in *E. coli* was produced in the manner described for synthesis of the gene for the C fragment of the A strand, namely, using  
20 a large number of oligomers of approximately 60-65 bases corresponding to the sequences of the <sub>+</sub> and <sub>-</sub> strands with overlaps of 7 bases. The oligomers were allowed to anneal and were ligated to form subunits of 250-300 base pairs each. Each subunit had been designed to have restriction sites at their termini which allowed them to be assembled in the right order to form the complete gene. the  
25 synthetic gene for encoding the c fragment of the B toxin was as follows:

ATGGCTTTCAACAAATACAATTCCGAAATCCTGAACAATATCATC  
CTGAACCTGCGTTACAAAGACAACAATCTGATCGATCTGTCTGGTTACGG  
TGCTAAAGTTGAAGTATACGACGGTGTGAACTGAATGACAAGAACCAG  
TTCAAAGTACCTCTTCCGCTAACTCTAAGATCCGTGTTACTCAGAATCA  
30 GAACATCATCTTCAACTCCGTATTCCTGGACTTCTCTGTTTCCTTCTGGAT  
CCGTATCCCGAAATACAAGAACGACGGTATCCAGAATTACATCCACAAT  
GAATACACCATCATCAACTGCATGAAGAATAACTCTGGTTGGAAGATCT

CCATCCGCGGTAACCGTATCATCTGGACTCTGATCGATATCAACGGTAAG  
ACCAAATCTGTATTCTTCGAATACAACATCCGTGAAGACATCTCTGAATA  
CATCAATCGCTGGTTCTTCGTTACCATCACCAATAACCTGAACAATGCTA  
AAATCTACATCAACGGTAAACTGGAATCTAATACCGACATCAAAGACAT  
5 CCGTGAAGTTATCGCTAACGGTGAAATCATCTTCAAACCTGGACGGTGAC  
ATCGATCGTACCCAGTTCATCTGGATGAAATACTTCTCCATCTTCAACAC  
CGAACTGTCTCAGTCCAATATCGAAGAACGGTACAAGATCCAGTCTTAC  
TCCGAATACCTGAAAGACTTCTGGGGTAATCCGCTGATGTACAACAAAG  
AATACTATATGTTCAATGCTGGTAACAAGAACTCTTACATCAAACCTGAA  
10 GAAAGACTCTCCGGTTGGTGAATCCTGACTCGTTCCAAATACAACCAGA  
ACTCTAAATACATCAACTACCGCGACCTGTACATCGGTGAAAAGTTCATC  
ATCCGTCGCAAATCTAACTCTCAGTCCATCAATGAGACATCGTACGTAAA  
GAAGACTACATCTACCTGGACTTCTTCAACCTGAATCAGAATGGCGTGTA  
TACACCTACAAGTACTTCAAGAAAGAAGAAGAAAAGCTTTTCCTGGCTC  
15 CGATCTCTGATTCCGACGAACTCTACAACACCATCCAGATCAAAGAATA  
CGACGAACAGCCGACCTACTCTTGCCAGCTGCTGTTCAAGAAAGATGAA  
GAATCTACTGACGAAATCGGTCTGATCGGTATCCACCGTTTCTACGAATC  
TGGTATCGTATTCTGAAGAATACAAAGACTCTTCTGCATCTCCAAATGGTA  
CCTGAAGGAAGTTAAACGCAAACCGTACAACCTGAAACTGGGTTGCAAT  
20 TGGCAGTTCATCCCGAAAGACGAAGGTTGGACCGAATAGTAACCTCTAG  
AGTCGAGGCCTGCAG

#### Cloning:

Supernatants of sonicated, IPTG-induced recombinant pMAL fusion *E. coli* cultures were tested for the presence of the botulinum H<sub>C</sub> expression product by  
25 ELISA and SDS-PAGE gels stained with coomassie brilliant blue were unsuccessful. Attempts to express H<sub>C</sub> fragment as a non-fusion product were unsuccessful. Initial characterization of plasmid DNA from putative clones in pKK233-2 demonstrated an insert of the expected size was present. In addition, SDS-PAGE indicated the presence of a protein of approximately 50 kDa after  
30 induction. However, the recombinants appeared unstable and further preparations of this and other cultures failed to reproduce these results. This approach was subsequently abandoned in favor of the fusion product expression.

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### Example 9 Immunization Trials:

Although attempts to quantitate expressed H<sub>C</sub> fusion products were unsuccessful, limited immunization trials were performed on mice to evaluate the vaccine potential of the product. Initial vaccination employed concentrated, crude  
5 *E. coli* lysate with complete Freund's adjuvant. Two weeks later, animals were boosted with amylose column-purified expression product with Freund's incomplete adjuvant. At this time, a second group of five animals received amylose purified product in Freund's incomplete adjuvant as a single vaccination. After two additional weeks, both groups were challenged intraperitoneally with a dose of 3  
10 LD<sub>50</sub> of toxin. All eleven animals receiving two immunizations with H<sub>C</sub> survived while six of the twelve control animals receiving pMAL vector alone died. Likewise, all five animals receiving one H<sub>C</sub> vaccination survived while animals receiving the pMAL vector alone died.

Four weeks after the initial challenge with 3 LD<sub>50</sub> of toxin, nine of the eleven  
15 animals who had received two immunizations were exposed to 30, 300, or 1200 LD<sub>50</sub> doses of toxin. The animals succumbing to the toxin challenge of 30 and 300 LD<sub>50</sub> did not exhibit fatality typical of botulinum toxin poisoning in that they appeared healthy after 18 hours, but were dead a few hours thereafter. In contrast, the animal which died from the 1200 LD<sub>50</sub> dose appeared moribund when examined  
20 at 18 hours and remained so until death. This reaction is consistent with symptoms usually observed with botulinum toxin-induced paralysis. Additional data on second challenge is shown on Table 5. Hence, it was shown that immunization with the genetically engineered toxin protected against large doses of the toxin.

It is also possible to produce antibodies using the genetically  
25 engineered toxin. Because the toxin is not disease-producing in the animal, it is possible to produce large amounts of antitoxin more cheaply. It is also possible to produce antitoxin using hybridoma technology.

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**TABLE 5**  
**PROTECTION OF MICE IMMUNIZED WITH H<sub>C</sub> OF A TOXIN**  
**DERIVED FROM SYNTHETIC GENE (# of deaths/total animals)**

Calculated challenge	Control	Protected
dose (LD <sub>50</sub> )	(vector without insert)	(Vector with insert)
4	2/3	0/3
10		0/3
30	3/4	0/3
100		0/3
300		0/3
1000		0/3
3000	0/1	

- 5 The animals received vaccinations of crude lysated cell material at 0, 2 and 4 weeks. Challenges were administered intraperitoneally with serotype A toxin at 5 weeks.

**Example 10 rBoNTA(H<sub>C</sub>) purification and protective effect**

- 10 Recombinant BoNTA(H<sub>C</sub>) peptide was produced recombinantly in yeast. The first step in the purification process for BoNTA(H<sub>C</sub>) was a Streamline expanded bed chromatography column. The product was eluted by a sodium chloride step gradient. Product eluted from the expanded bed chromatography column was estimated to be 10% pure with a total protein concentration of 0.92 mg/ml. After dialyzing the salt away, the material was loaded onto a mono S cation exchange column for further purification.
- 15 Western blot and ELISA data indicated that BoNTA(H<sub>C</sub>) eluted from the column at 110 mM sodium chloride. The Mono S pool was subjected to HIC as a final purification step and thus, the material was adjusted to 1.5 M ammonium sulfate. The Mono S product was loaded onto a HIC column and eluted with a gradient of decreasing ammonium sulfate. Product eluted at 1.04 M ammonium sulfate and BoNTA(H<sub>C</sub>) immunologically positive
- 20 fractions were combined and dialyzed to remove ammonium sulfate. Only a 50 kDa BoNTA(H<sub>C</sub>) band was detected by SDS-PAGE and Western blot analysis and was judged to be greater than 95% pure after the final step. Protective effect of this purified material was measured by immunizing mice with 1 dose followed by challenge with 1000 LD<sub>50</sub> of BoNTA(H<sub>C</sub>). The results are shown in Table 6 below.

**Table 6 Potency assay: 1 dose followed by challenge with 1000 LD50 of BoNTA(H<sub>C</sub>)**

Dose (μg)	survival
10	10/10
2.5	10/10
0.625	10/10
0.156	7/10
0.039	2/10
0.0098	0/10
0.0024	0/10

**Example 11 rBoNTB(H<sub>C</sub>) purification and protective effect**

5 Recombinant BoNTC(H<sub>C</sub>) peptide was produced recombinantly in yeast. The first separation technique employed for the purification process for BoNTB(H<sub>C</sub>) was Streamline chromatography (Pharmacia), which is a single pass expanded bed adsorption operation where proteins can be recovered from crude feed stock or cell lysate without prior clarification. Significant clean-up was accomplished in this step as the MES buffer system prohibited binding of a large percentage of unwanted proteins to the SP resin. Protein was loaded onto the column at a concentration of 123 mg/mL-resin, using 20 mM MES buffer, pH 5.7 with 10 mM NaCl. The product pool was eluted in a single step. Under the conditions investigated, on average 3.9% of the total protein loaded was recovered in the elution peak, and the product pool was approximately 70% BoNTB(H<sub>C</sub>) fragment based on SDS-PAGE.

The second chromatography step in the process utilizes Poros HS, another strong cation exchange resin. The buffer system was similar to that used for Streamline SP, however enhanced selectivity of Poros HS enriched the product peak to about 85% purity. The product peak eluted during the gradient at approximately 130 mM NaCl. Strongly bound proteins were eluted with 1 M NaCl.

The final chromatography step utilized a Poros PI column. Analysis of the PI fractions by SDS-PAGE and IEF revealed that the product band, a single band at 50 kD

on SDS-PAGE, was present in the pH 8.0 fraction. Analysis of purified BoNTB(H<sub>C</sub>) fragment by 2-D electrophoresis resulted in one major spot and two minor, faint spots from the PI-peak 1 fraction. Peak 2 contained several spots at two different molecular weights corresponding to 50 kD and 47 kD. Presumably these spots represent different isoforms. IEF banding patterns detected in the first dimension are in agreement with those seen in Phast IEF for the two peaks. The protective efficacy of this material was determined by potency assay of 1 dose followed by challenge with 1000 LD<sub>50</sub> of BoNTB(H<sub>C</sub>). The results are shown in the following Table 7.

**Table 7      BoNTB(H<sub>C</sub>)**

Dose (μg)	survival
10	10/10
2.5	10/10
0.625	10/10
0.156	6/10
0.039	1/10
0.0098	0/10
0.0024	0/10

**Example 12    rBoNTC(H<sub>C</sub>) purification and protective effect**

Recombinant BoNTC(H<sub>C</sub>) peptide was produced recombinantly in yeast. The initial chromatography step used for the purification process for BoNTC<sub>1</sub>(H<sub>C</sub>) was a Mono Q anion-exchange column. The column was equilibrated with 50 mM sodium phosphate, 0.2% (W/V) CHAPS, 2 mM EDTA, pH 7.0. The CHAPS was incorporated into the column buffers to allow product to elute from the column over a narrower sodium chloride concentration. Fractions positive for BoNTC<sub>1</sub>(H<sub>C</sub>) by Western analysis were pooled and adjusted to 1 M ammonium sulfate. A moderate precipitate formed which was removed by passing the material through a 0.2 μ filtration unit. The clarified Mono Q product pool was subjected to hydrophobic interaction chromatography using a Pharmacia alkyl superose column. This final step removed the remainder of the impurities liberating BoNTC<sub>1</sub>(H<sub>C</sub>) product which was estimated to be greater than 98% pure as judged by SDS/PAGE. Protective effect of this purified material was measured by immunizing mice

with 1 dose followed by challenge with 1000 LD50 of BoNTC<sub>1</sub>(H<sub>C</sub>). The results are shown in Table 8 below.

**Table 8. Potency Assay: One dose followed by challenge with 1000 LD50 of BoNTC<sub>1</sub>(H<sub>C</sub>)**

Dose (μg)	Survival
8.1	10/10
2.7	10/10
0.9	10/10
0.3	9/10
0.1	4/10
0.033	0/10
0.011	0/10

5

For purposes of clarity of understanding, the foregoing invention has been described in some detail by way of illustration and example in conjunction with specific embodiments, although other aspects, advantages and modifications will be apparent to those skilled in the art to which the invention pertains. The foregoing description and examples are intended to illustrate, but not limit the scope of the invention. Modifications of the above-described modes for carrying out the invention that are apparent to persons of skill in medicine, immunology, hybridoma technology, pharmacology, and/or related fields are intended to be within the scope of the invention, which is limited only by the appended claims.

All publications and patent applications mentioned in this specification are indicative of the level of skill of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

20

**CLAIMS:**

1. A nucleic acid encoding the carboxy-terminal portion of the heavy chain (H<sub>C</sub>) of botulinum neurotoxin (BoNT) selected from the group consisting of BoNT serotype A, BoNT serotype B, BoNT serotype C<sub>1</sub>, BoNT serotype D, BoNT serotype E, BoNT serotype F, and BoNT serotype G, wherein said nucleic acid is expressable in a recombinant organism selected from *Escherichia coli* and *Pichia pastoris*.  
5
2. The nucleic acid of claim 1, wherein said nucleic acid comprises a nucleic acid sequence selected from SEQ ID No:1 (serotype A), SEQ ID No:7 (serotype B), SEQ ID No:9 (serotype C<sub>1</sub>), SEQ ID No:11 (serotype D), SEQ ID No:13 (serotype E), SEQ ID No:15 (serotype F), and SEQ ID No:17 (serotype G).  
10
3. The nucleic acid of claim 1, wherein the nucleic acid encodes an H<sub>C</sub> amino acid sequence of BoNT selected from SEQ ID No:2 (serotype A), SEQ ID No:8 (serotype B), SEQ ID No:10 (serotype C<sub>1</sub>), SEQ ID No:12 (serotype D), SEQ ID No:14 (serotype E), SEQ ID No:16 (serotype F), and SEQ ID No:18 (serotype G).  
15
4. A nucleic acid encoding the amino-terminal portion of the heavy chain (H<sub>N</sub>) of botulinum neurotoxin (BoNT) selected from the group consisting of BoNT serotype B, BoNT serotype C<sub>1</sub>, BoNT serotype D, BoNT serotype E, BoNT serotype F, and BoNT serotype G, wherein said nucleic acid is expressable in a recombinant organism selected from *Escherichia coli* and *Pichia pastoris*.  
20
5. The nucleic acid of claim 4, wherein said nucleic acid comprises a nucleic acid sequence selected from SEQ ID No:21 (serotype B), SEQ ID No:23 (serotype C<sub>1</sub>), SEQ ID No:25 (serotype D), SEQ ID No:27 (serotype E), SEQ ID No:29 (serotype F), and SEQ ID No:31 (serotype G).  
25
6. The nucleic acid of claim 4, wherein the nucleic acid encodes an H<sub>N</sub> amino acid sequence of BoNT selected from SEQ ID No:22 (serotype B), SEQ ID No:24 (serotype C<sub>1</sub>), SEQ ID No:26 (serotype D), SEQ ID No:28 (serotype E), SEQ ID No:30 (serotype F), and SEQ ID No:32 (serotype G).  
30

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7. The nucleic acid of any one of claims 1, 3, 4, or 6, wherein the sequence of the nucleic acid is designed by selecting at least a portion of the codons encoding H<sub>C</sub> from codons preferred for expression in a host organism.

5

8. The nucleic acid of claim 7, wherein the host organism is selected from gram negative bacteria, yeast, and mammalian cell lines.

9. The nucleic acid of claim 8, wherein the host organism is *Escherichia coli* or  
10 *Pichia pastoris*.

10. The nucleic acid of any one of claims 1, 3, 4, or 6, wherein the nucleic acid sequence encoding H<sub>C</sub> is designed by selecting codons encoding H<sub>C</sub> which codons provide H<sub>C</sub> sequence enriched in guanosine and cytosine residues.

15

11. The nucleic acid of any one of claims 1, 3, 4, or 6, wherein said nucleic acid is a synthetic nucleic acid.

12. The nucleic acid of any one of claims 1, 3, 4, or 6, wherein said nucleic acid  
20 encoding H<sub>C</sub> or H<sub>N</sub> is expressed in a recombinant host organism with higher yield than a second nucleic acid fragment encoding the same H<sub>C</sub> sequence, said second nucleic acid fragment having the wild-type *Clostridium botulinum* sequence of H<sub>C</sub>.

13. An expression vector comprising the nucleic acid of any one of claims 1, 3,  
25 4, or 6, whereby H<sub>C</sub> or H<sub>N</sub> is expressed upon transfection of a host organism with said expression vector.

14. A method of preparing a polypeptide comprising the carboxy-terminal  
30 portion of the heavy chain (H<sub>C</sub>) of botulinum neurotoxin (BoNT) or the amino-terminal portion of the heavy chain (H<sub>N</sub>) of botulinum neurotoxin (BoNT) selected from the group consisting of BoNT serotype A, BoNT serotype B, BoNT serotype C, BoNT serotype D, BoNT serotype E, BoNT serotype F, and BoNT serotype G,

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said method comprising culturing a recombinant host organism transfected with the expression vector of claim 13 under conditions wherein  $H_C$  or  $H_N$  is expressed.

15     15.     The method of claim 14, wherein the recombinant host organism is a eukaryote.

16.     The method of claim 14, further comprising recovering insoluble protein from said host organism, whereby a fraction enriched in  $H_C$  or  $H_N$  is obtained.

10     17.     The method of claim 16, wherein said host organism is *Pichia pastoris*.

15     18.     An immunogenic composition comprising the carboxy-terminal portion of the heavy chain ( $H_C$ ) of botulinum neurotoxin (BoNT) selected from the group consisting of BoNT serotype A, BoNT serotype B, BoNT serotype C, BoNT serotype D, BoNT serotype E, BoNT serotype F, and BoNT serotype G.

20     19.     The immunogenic composition of claim 18, wherein  $H_C$  is prepared by culturing a recombinant organism transfected with an expression vector encoding  $H_C$ .

20.     The immunogenic composition of claim 19, wherein an insoluble protein fraction enriched in  $H_C$  is recovered from said recombinant organism.

25     21.     An immunogenic composition comprising the amino-terminal portion of the heavy chain ( $H_N$ ) of botulinum neurotoxin (BoNT) selected from the group consisting of BoNT serotype A, BoNT serotype B, BoNT serotype C, BoNT serotype D, BoNT serotype E, BoNT serotype F, and BoNT serotype G.

30     22.     The immunogenic composition of claim 21, wherein  $H_N$  is prepared by culturing a recombinant organism transfected with an expression vector encoding  $H_N$ .

23. The immunogenic composition of claim 22, wherein an insoluble protein fraction enriched in H<sub>N</sub> is recovered from said recombinant organism.

24. An immunogenic composition comprising a polypeptide comprising epitopes  
5 contained in the carboxy-terminal portion of the heavy chain (H<sub>C</sub>) of botulinum neurotoxin (BoNT) or the amino-terminal portion of the heavy chain (H<sub>N</sub>) of botulinum neurotoxin (BoNT) selected from the group consisting of BoNT serotype A, BoNT serotype B, BoNT serotype C, BoNT serotype D, BoNT serotype E, BoNT serotype F, and BoNT serotype G, said epitopes eliciting protective immunity  
10 toward the respective BoNT serotype.

25. The immunogenic composition of claim 24, wherein said immunogenic composition elicits an ELISA response to the respective BoNT serotype in an animal, said ELISA response being detectable upon 100-fold dilution of serum from  
15 said animal.

26. An immunogenic composition comprising a protein containing at least a portion of a botulinum neurotoxin (BoNT) sequence, said BoNT being selected from the group consisting of BoNT serotype A, BoNT serotype B, BoNT serotype C,  
20 BoNT serotype D, BoNT serotype E, BoNT serotype F, and BoNT serotype G.

27. The immunogenic composition of claim 26, wherein said portion of BoNT sequence elicits an ELISA response to the respective BoNT serotype in an animal, said ELISA response being detectable upon 100-fold dilution of serum from said  
25 animal.

28. The immunogenic composition of claim 26, wherein said protein is a fusion protein further comprising a non-toxic polypeptide sequence.

29. The immunogenic composition of claim 26, wherein said composition is  
30 endotoxin free.

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30. The immunogenic composition of claim 26, wherein the portion of BoNT sequence is a portion of carboxy-terminal heavy chain (H<sub>C</sub>) sequence or amino-terminal heavy chain (H<sub>N</sub>) sequence.

5 31. A nucleic acid encoding a protein containing at least a portion of a botulinum neurotoxin (BoNT) sequence, said BoNT being selected from the group consisting of BoNT serotype A, BoNT serotype B, BoNT serotype C, BoNT serotype D, BoNT serotype E, BoNT serotype F, and BoNT serotype G.

10 32. The nucleic acid of claim 31, wherein said protein containing at least a portion of BoNT sequence elicits an ELISA response to the respective BoNT serotype in an animal, said ELISA response being detectable upon 100-fold dilution of serum from said animal.

15 33. An expression vector comprising the nucleic acid of claim 31.

34. A recombinant host cell containing the expression vector of claim 33.

20 35. The recombinant host cell of claim 34, wherein said host cell expresses a protein containing at least a portion of BoNT sequence, said portion of BoNT sequence containing at least one protective epitope of the respective BoNT serotype.

25 36. The recombinant host cell of claim 34, wherein said host cell expresses a protein containing at least a portion of BoNT sequence, and wherein said protein elicits an ELISA response to the respective BoNT serotype in an animal, said ELISA response being detectable upon 100-fold dilution of serum from said animal.

30 37. The recombinant host cell of claim 34, wherein said host cell expresses a protein containing at least a portion of BoNT sequence, said protein making up at least 0.75% of the total cellular protein.

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38. The recombinant host cell of claim 34, wherein said host cell expresses a protein containing at least a portion of BoNT sequence, said protein making up at least 20% of the total cellular protein.

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## ABSTRACT

This invention is directed to preparation and expression of synthetic genes encoding polypeptides containing protective epitopes of botulinum neurotoxin (BoNT). The invention is also directed to production of immunogenic peptides encoded by the synthetic genes, as well as recovery and purification of the immunogenic peptides from recombinant organisms. The invention is also directed to methods of vaccination against botulism using the expressed peptides.

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FIGURE 1

**A: BoNTA(H<sub>c</sub>) synthetic gene (Version #1) SEQ ID NO: 1**

GAATTCGAAACGatgCGTCTGCTGTCTACCTTCACTGAATACATCAAGAAC  
ATCATCAATACCTCCATCCTGAACCTGCGCTACGAATCCAATCACCTGAT  
CGACCTGTCTCGCTACGCTTCCAAAATCAACATCGGTTCTAAAGTAACT  
TCGATCCGATCGACAAGAATCAGATCCAGCTGTTCAATCTGGAATCTTCC  
AAAATCGAAGTTATCCTGAAGAATGCTATCGTATACAACTCTATGTACG  
AAAACTTCTCCACCTCCTTCTGGATCCGTATCCCGAAATACTTCAACTCC  
ATCTCTCTGAACAATGAATACACCATCATCAACTGCATGGAAAACAATT  
CTGGTTGGAAAGTATCTCTGAACTACGGTGAAATCATCTGGACTCTGCAG  
GACACTCAGGAAATCAAACAGCGTGTGATTCAAATACTCTCAGATGA  
TCAACATCTCTGACTACATCAATCGCTGGATCTTCGTTACCATCACCAAC  
AATCGTCTGAATAACTCCAAAATCTACATCAACGGCCGTCTGATCGACC  
AGAAACCGATCTCCAATCTGGGTAACATCCACGCTTCTAATAACATCATG  
TTCAAACCTGGACGGTTGTCGTGACACTCACCGCTACATCTGGATCAAATA  
CTTCAATCTGTTTCGACAAAGAACTGAACGAAAAAGAAATCAAAGACCTG  
TACGACAACCGAGTCCAATTCTGGTATCCTGAAAGACTTCTGGGGTGACTA  
CCTGCAGTACGACAAACCGTACTACATGCTGAATCTGTACGATCCGAAC  
AAATACGTTGACGTCAACAATGTAGGTATCCGCGGTTACATGTACCTGA  
AAGGTCCGCGTGGTTCTGTTATGACTACCAACATCTACCTGAACTCTTCC  
CTGTACCGTGGTACCAAATTCATCATCAAGAAATACGCGTCTGGTAACA  
AGGACAATATCGTTCGCAACAATGATCGTGTATACATCAATGTTGTAGTT  
AAGAACAAAGAATACCGTCTGGCTACCAATGCTTCTCAGGCTGGTGTAG  
AAAAGATCTTGTCTGCTCTGGAAATCCCGGACGTTGGTAATCTGTCTCAG  
GTAGTTGTAATGAAATCCAAGAACGACCAGGGTATCACTAACAATGCA  
AAATGAATCTGCAGGACAACAATGGTAACGATATCGGTTTCATCGGTTT  
CCACCAGTTCAACAATATCGCTAAACTGGTTGCTTCCAACCTGGTACAATC  
GTCAGATCGAACGTTCCCTCTCGCACTCTGGGTTGCTCTTGGGAGTTCATC  
CCGGTTGATGACGGTTGGGGTGAACGTCCGCTGtaaGAATTC

**B: BoNTA(H<sub>c</sub>) encoded protein (Version #1) SEQ ID NO: 2**

MRLSTFTEYIKNIINTSILNLRYESNHLIDLSRYASKINIGSKVNFDPIDKNQI  
QLFNLESSKIEVILKNAIVYNSMYENFSTSFWRIPKYFNSISLNNEYTIINCME  
NNSGWKVSLNYGEIIWTLQDTQEIQRVVFKYSQMINISDYINRWIFVTITNN  
RLNNSKIYINGRLIDQKPISNLGNIHASNNIMFKLDGCRDTHRYIWIKYFNLF  
DKELNEKEIKDLYDNQNSNGILKDFWGDYLQYDKPYMLNLYDPNKYVDV  
NNVGIRGYMYLKGPRGSVMTTNIYLNSSLYRGTKFIIKKYASGNKDNIVRN  
NDRVYINVVVKNKEYRLATNASQAGVEKILSALEIPDVGNLSQVVVMKSKN  
DQGITNKCKMNLQDNNNGNDIGFIGFHQFNIAKLVASNWYNRQIERSRRTL  
GCSWEFIPVDDGWGERPL\*

FIGURE 2

**A: BoNTA(H<sub>c</sub>) synthetic gene (version #2) SEQ ID NO: 3**

GAATTCGAAACGatgTCTACCTTCACTGAATACATCAAGAACATCATCAAT  
ACCTCCATCCTGAACCTGCGCTACGAATCCAATCACCTGATCGACCTGTC  
TCGCTACGCTTCCAAAATCAACATCGGTTCTAAAGTTAACTTCGATCCGA  
TCGACAAGAATCAGATCCAGCTGTTCAATCTGGAATCTTCCAAAATCGA  
AGTTATCCTGAAGAATGCTATCGTATACAACCTCTATGTACGAAAACCTCT  
CCACCTCCTTCTGGATCCGTATCCCGAAATACTTCAACTCCATCTCTCTG  
AACAATGAATACACCATCATCAACTGCATGGAAAACAATTCTGGTTGGA  
AAGTATCTCTGAACTACGGTGAAATCATCTGGACTCTGCAGGACACTCA  
GGAAATCAAACAGCGTGTTGTATTCAAATACTCTCAGATGATCAACATCT  
CTGACTACATCAATCGCTGGATCTTCGTTACCATCACCAACAATCGTCTG  
AATAACTCCAAAATCTACATCAACGGCCGCTCTGATCGACCAGAAACCGA  
TCTCCAATCTGGGTAAACATCCACGCTTCTAATAACATCATGTTCAAACCTG  
GACGGTTGTCGTGACACTACCGCTACATCTGGATCAAATACTTCAATCT  
GTTTCGACAAAGAACTGAACGAAAAAGAAATCAAAGACCTGTACGACAA  
CCAGTCCAATTCTGGTATCCTGAAAGACTTCTGGGGTGACTACCTGCAGT  
ACGACAAACCGTACTACATGCTGAATCTGTACGATCCGAACAAATACGT  
TGACGTCAACAATGTAGGTATCCGCGGTTACATGTACCTGAAAGGTCCG  
CGTGGTTCTGTTATGACTACCAACATCTACCTGAACTCTTCCCTGTACCG  
TGGTACCAAATTCATCATCAAGAAATACGCGTCTGGTAACAAGGACAAT  
ATCGTTCGCAACAATGATCGTGTATACATCAATGTTGTAGTTAAGAACAA  
AGAATACCGTCTGGCTACCAATGCTTCTCAGGCTGGTGTAGAAAAGATC  
TTGTCTGCTCTGGAAATCCCGGACGTTGGTAATCTGTCTCAGGTAGTTGT  
AATGAAATCCAAGAACGACCAGGGTATCACTAACAATGCAAAATGAAT  
CTGCAGGACAACAATGGTAACGATATCGGTTTCATCGGTTTCCACCAGTT  
CAACAATATCGCTAAACTGGTTGCTTCCAACCTGGTACAATCGTCAGATCG  
AACGTTCCCTCTCGCACTCTGGGTTGCTCTTGGGAGTTCATCCCGGTTGAT  
GACGGTTGGGGTGAACGTCCGCTGtaaGAATTC

**B: BoNTA(H<sub>c</sub>) encoded protein (Version #2) SEQ ID NO: 4**

MSTFTEYIKNIINTSILNLRYESNHLIDLSRYASKINIGSKVNFDPIDKNQIQLF  
NLESSKIEVILKNAIVYNSMYENFSTSFWIRIPKYFNISLNNNEYTIINCMENNS  
GWKVSILNYGEIHWTLQDTQEIKQRVVFKEYSQMINISDYINRWIFVTITNNRLN  
NSKIYINGRLIDQKPISNLGNIHASNNIMFKLDGCRDTHRYTWIKYFNLFDFKE  
LNEKEIKDLYDNQSNNGILKDFWGDYLYDKPYMLNLYDPNKYVDVNNV  
GIRGYMYLKGPRGSVMTTNIYLNSSLYRGTKFIIKKYASGNKDNIVRNNDRV  
YINVVVKNKEYRLATNASQAGVEKILSALEIPDVGNLSQVVVMKSKNDQGI  
TNKCKMNLQDNNGNDIGFIGFHQFNIAKLVASNWYNRQIERSSRTLGCWSW  
EFIPVDDGWGERPL\*



FIGURE 3

**A: BoNTA(H<sub>2</sub>) synthetic gene (Version #3) SEQ ID NO: 5**

GAATTCGAAACGatgGCCTCTACCTTCACTGAATACATCAAGAACATCATC  
AATACCTCCATCCTGAACCTGCGCTACGAATCCAATCACCTGATCGACCT  
GTCTCGCTACGCTTCCAAAATCAACATCGGTTCTAAAGTTAACTTCGATC  
CGATCGACAAGAATCAGATCCAGCTGTTCAATCTGGAATCTTCCAAAAT  
CGAAGTTATCCTGAAGAATGCTATCGTATACAACCTCTATGTACGAAAAC  
TCTCCACCTCCTTCTGGATCCGTATCCCGAAATACTTCAACTCCATCTCTC  
TGAACAATGAATACACCATCATCAACTGCATGGAAAACAATTCTGGTTG  
GAAAGTATCTCTGAACTACGGTGAAATCATCTGGACTCTGCAGGACACT  
CAGGAAATCAAACAGCGTGTTGTATTCAAATACTCTCAGATGATCAACA  
TCTCTGACTACATCAATCGCTGGATCTTCGTTACCATCACCAACAATCGT  
CTGAATAACTCCAAAATCTACATCAACGGCCGTCTGATCGACCAGAAAC  
CGATCTCCAATCTGGGTAACATCCACGCTTCTAATAACATCATGTTCAAA  
CTGGACGGTTGTCGTGACACTCACCGCTACATCTGGATCAAATACTTCAA  
TCTGTTTCGACAAAGAACTGAACGAAAAAGAAATCAAAGACCTGTACGAC  
AACCAGTCCAATTCTGGTATCCTGAAAGACTTCTGGGGTGACTACCTGCA  
GTACGACAAACCGTACTACATGCTGAATCTGTACGATCCGAACAAATAC  
GTTGACGTCAACAATGTAGGTATCCGCGGTTACATGTACCTGAAAGGTC  
CGCGTGGTTCTGTTATGACTACCAACATCTACCTGAACTCTTCCCTGTAC  
CGTGGTACCAAATTCATCATCAAGAAATACGCGTCTGGTAACAAGGACA  
ATATCGTTCGCAACAATGATCGTGTATACATCAATGTTGTAGTTAAGAAC  
AAAGAATACCGTCTGGCTACCAATGCTTCTCAGGCTGGTGTAGAAAAGA  
TCTTGTCTGCTCTGGAAATCCCGGACGTTGGTAATCTGTCTCAGGTAGTT  
GTAATGAAATCCAAGAACGACCAGGGTATCACTAACAAATGCAAAATGA  
ATCTGCAGGACAACAATGGTAACGATATCGGTTTCATCGGTTTCCACCAG  
TTCAACAATATCGCTAAACTGGTTGCTTCCAACCTGGTACAATCGTCAGAT  
CGAACGTTCTCTCGCACTCTGGGTTGCTCTTGGGAGTTCATCCCGGTTG  
ATGACGGTTGGGGTGAACGTCCGCTGtaaGAATTC

**B: BoNTA(H<sub>2</sub>) encoded protein (Version #3) SEQ ID NO: 6**

MASTFTEYIKNIINTSILNLRYESNHLIDLRSYASKINIGSKVNFDPIDKNQIQL  
FNLESSKIEVILKNAIVYNSMYENFSTSWIRIPKYFNSSISLNNEYTIINCMENN  
SGWKVSLNYGEIHWTLQDTQEIQRVVFKYSQMINISDYINRWIFVTITNNRL  
NNSKIYINGRLIDQKPISNLGNIHASNNIMFKLDGCRDTHRYIWIKYFNLFDK  
ELNEKEIKDLYDNQSNISGILKDFWGDYLYDKPYMLNLYDPNKYVDVNN  
VGIRGYMYLKGPRGSVMTTNIYLNSSLYRGTKFIIKKYASGNKDNIVRNNDR  
VYINVVVKNKEYRLATNASQAGVEKILSALEIPDVGNLSQVVVMKSKNDQ  
GITNKCKMNLQDNNGNDIGFIGFHQFNNAIKLVASNWYNRQIERSSRTLGC  
SWEFIPVDDGWGERPL\*

FIGURE 4

**A: BoNTB(H<sub>c</sub>) synthetic gene SEQ ID NO: 7**

GAATTCACGatgGCCAACAAATACAATTCCGAAATCCTGAACAATATCAT  
CCTGAACCTGCGTTACAAAGACAACAATCTGATCGATCTGTCTGGTTACG  
GTGCTAAAGTTGAAGTATACGACGGTGTGAACTGAATGACAAGAACCA  
GTTCAAACCTGACCTCTTCCGCTAACTCTAAGATCCGTGTTACTCAGAATC  
AGAACATCATCTTCAACTCCGTATTCTGGACTTCTCTGTTTCCTTCTGGA  
TTCGTATCCCGAAATACAAGAACGACGGTATCCAGAATTACATCCACAA  
TGAATACACCATCATCAACTGCATGAAGAATAACTCTGGTTGGAAGATC  
TCCATCCGCGGTAACCGTATCATCTGGACTCTGATCGATATCAACGGTAA  
GACCAAATCTGTATTCTTCGAATACAACATCCGTGAAGACATCTCTGAAT  
ACATCAATCGCTGGTTCTTCGTTACCATCACCAATAACCTGAACAATGCT  
AAAATCTACATCAACGGTAAACTGGAATCTAATACCGACATCAAAGACA  
TCCGTGAAGTTATCGCTAACGGTGAAATCATCTTCAAACCTGGACGGTGA  
CATCGATCGTACCCAGTTCATCTGGATGAAATACTTCTCCATCTTCAACA  
CCGAACCTGTCTCAGTCCAATATCGAAGAACGGTACAAGATCCAGTCTTA  
CTCCGAATACCTGAAAGACTTCTGGGGTAATCCGCTGATGTACAACAAA  
GAATACTATATGTTCAATGCTGGTAACAAGAAGCTTACATCAAACCTGA  
AGAAAGACTCTCCGGTTGGTGAAATCCTGACTCGTTCCAAATACAACCA  
GAACTCTAAATACATCAACTACCGCGACCTGTACATCGGTGAAAAGTTC  
ATCATCCGTCGCAAATCTAACTCTCAGTCCATCAATGATGACATCGTACG  
TAAAGAAGACTACATCTACCTGGACTTCTTCAACCTGAATCAGGAATGG  
CGTGTATACACCTACAAGTACTTCAAGAAAGAAGAAGAAAAGCTTTTCC  
TGGCTCCGATCTCTGATTCCGACGAACTCTACAACACCATCCAGATCAAA  
GAATACGACGAACAGCCGACCTACTCTTGCCAGCTGCTGTTCAAGAAAG  
ATGAAGAATCTACTGACGAAATCGGTCTGATCGGTATCCACCGTTTCTAC  
GAATCTGGTATCGTATTCGAAGAATACAAGACTACTTCTGCATCTCCAA  
ATGGTACCTGAAGGAAGTTAAACGCAAACCGTACAACCTGAAACTGGGT  
TGCAATTGGCAGTTCATCCCGAAAGACGAAGGTTGGACCGAAtagtaaGAA  
TTC

**B: BoNTB(H<sub>c</sub>) encoded protein SEQ ID NO: 8**

MANKYNSEILNNIILNLRYKDNNLIDLSGYGAKVEVYDGVELNDKNQFKLT  
SSANSKIRVTQNQNIIFNSVFLDFSVSFWIRIPKYKNDGIQNYIHNEYTIINCM  
KNNSGWKISIRGNRIIWTLLIDINGKTKSVFFEYNIREDISEYINRWFFVTITNNL  
NNAKIYINGKLESNTDIKDIREVIANGEIIFKLDGDIDRTQFIWMKYFSIFNTEL  
SQSNIEERYKIQSYSEYLKDFWGNPLMYNKEYYMFNAGNKNSYIKLKKDSP  
VGEILTRSKYNQNSKYINYRDLIYGEKFIIRRSNSQSINDDIVRKEDIYILDF  
FNLNQEWVRVYTYKYFKKEEEKLFLAPISDSDELYNTIQIKEYDEQPTYSCQL  
LFKKDEESTDEIGLIGIHRFYESGIVFEEYKDYFCISKWYLKEVKRKPYNLKL  
GCNWQFIPKDEGWTE\*\*

FIGURE 5

**A: BoNTC<sub>1</sub>(H<sub>c</sub>) synthetic gene SEQ ID NO: 9**

GAATTCACGatgACCATCCCATTCAACATCTTCTCCTACACCAACAACCTCC  
CTGTTGAAGGACATCATCAACGAGTACTTCAACAACATCAACGACTCCA  
AGATCCTGTCCCTGCAGAACCGTAAGAACACCTTGGTCGACACCTCCGG  
TTACAACGCCGAGGTCTCCGAGGAGGGTGACGTCCAGCTGAACCCAATC  
TTCCCATTCGACTTCAAGCTGGGTTCCTCCGGTGAGGACAGAGGTAAGGT  
CATCGTCACCCAGAACGAGAACATCGTCTACAACCTCCATGTACGAGTCC  
TTCTCCATCTCCTTCTGGATCAGAATCAACAAGTGGGTCTCCAACCTGCC  
AGGTTACACCATCATCGACTCCGTCAAGAACAACCTCCGGTTGGTCCATCG  
GTATCATCTCCAACCTTCTGCTTCCACCTGAAGCAGAACGAGGACTCC  
GAGCAGTCCATCAACTTCTCCTACGACATCTCCAACAACGCTCCTGGTTA  
CAACAAGTGGTTCTTCGTCACCGTCACCAACAACATGATGGGTAAACATG  
AAGATCTACATCAACGGTAAGCTGATCGACACCATCAAGGTCAAGGAGT  
TGACCGGTATCAACTTCTCCAAGACCATCACCTTCGAGATCAACAAGATC  
CCAGACACCGGTCTGATCACCTCCGACTCCGACAACATCAACATGTGGA  
TCCGTGACTTCTACATCTTCGCCAAGGAGTTGGACGGTAAGGACATCAA  
CATCCTGTTCAACTCCTTGCAGTACACCAACGTCGTCAAGGACTACTGGG  
GTAACGACCTGAGATACAACAAGGAGTACTACATGGTCAACATCGACTA  
CTTGAACAGATACATGTACGCCAAGTCCAGACAGATCGTCTTCAACACC  
AGACGTAACAACAACGACTTCAACGAGGGTTACAAGATCATCATCAAGC  
GTATCAGAGGTAACACCAACGACACCAGAGTCAGAGGTGGTGACATCCT  
GTACTTCGACATGACTATCAACAACAAGGCCTACAACCTGTTTCATGAAG  
AACGAGACCATGTACGCCGACAACCACTCCACCGAGGACATCTACGCCA  
TCGGTCTGCGTGAGCAGACCAAGGACATCAACGACAACATCATCTTCCA  
GATCCAGCCAATGAACAACACTTACTACTACGCTTCCCAGATCTTCAAGT  
CCAACCTTCAACGGTGAGAACATCTCCGGTATCTGTTCCATCGGTACCTAC  
AGATTCCGTCTGGGTGGTGACTGGTACAGACACAACCTTGGTTCCAAC  
TGTC AAGCAGGGTA ACTACGCCTCCTTGCTGGAGTCCACTTCCACCCACT  
GGGGATTTCGTCCCAGTCTCCGAGtaatagGAATTC

**B: BoNTC<sub>1</sub>(H<sub>c</sub>) encoded protein SEQ ID NO: 10**

MTIPFNIFS YTNNSLLKDIINEYFNNINDSKILSLQNRKNTLVDTSGYNAEVSE  
EGDVQLNPIFPDFKLGSSGEDRGKVIVTQENIVYNSMYESFSISFWIRINK  
WVSNLPGYTIIDSVKNNSGWSIGIISNFLVFTLKQNEDEQSINFSYDISNNAP  
GYNKWFFVTVTNNMMGNMKIYINGKLIDTIKVKELTGINFSTITFEINKIPD  
TGLITSDSDNINMWIRDFYIFAKELDGKDINILFNSLQYTNVVKDYWGNDLR  
YNKEYYMVNIDYLNRYMYANSRQIVFNTRNNNDFNEGKIIKRIRGNTN  
DTRVRGGDILYFDMTINNKAYNLFMKNETMYADNHSTEDIYAIGLREQTKD  
INDNIIFQIQPMNNTYYYASQIFKSNFNGENISGICSIGTYRFRLGGDWYRHN  
YLVP TVKQGN YASLLESTSTHWGFVPVSE\*\*

FIGURE 6

**A: BoNTD(H<sub>o</sub>) synthetic gene SEQ ID NO: 11**

GAATTCACGatgCGTTTGAAGGCTAAGGTCAACGAGTCCTTCGAGAACAC  
CATGCCATTCAACATCTTCTCCTACACCAACAACCTCCTTGTTGAAGGACA  
TCATCAACGAGTACTTCAACTCCATCAACGACTCCAAGATCTTGTCTTG  
CAGAACAAGAAGAACGCCTTGGTTCGACACCTCCGGTTACAACGCCGAGG  
TCAGAGTCGGTGACAACGTCCAGTTGAACACCATCTACACCAACGACTT  
CAAGTTGTCTCTTCCGGTGACAAGATCATCGTCAACTTGAACAACAACA  
TCTTGTAATCCGCCATCTACGAGAACTCCTCTGTCTCCTTCTGGATCAAG  
ATCTCCAAGGACTTGACCAACTCCCACAACGAGTACACCATCATCAACT  
CCATCGAGCAGAACTCCGGTTGGAAGTTGTGTATCCGTAACGGTAACAT  
CGAGTGGATCTTGCAGGACGTCAACCGTAAGTACAAGTCCTTGATCTTCG  
ACTACTCCGAGTCCTTGTCCCACACCGGTTACACCAACAAGTGGTTCTTC  
GTCACCATCACCAACAACATCATGGGTACATGAAGTTGTACATCAACG  
GTGAGTTGAAGCAGTCCCAGAAGATCGAGGACCTGGACGAGGTCAAGCT  
GGACAAGACCATCGTCTTCGGTATCGACGAGAACATCGACGAGAACCAG  
ATGTTGTGGATCCGTGACTTCAACATCTTCTCCAAGGAGCTGTCCAACGA  
GGACATCAACATCGTCTACGAGGGTCAGATCCTGAGGAACGTCATCAAG  
GACTACTGGGGTAACCCACTGAAGTTCGACACCGAGTACTACATCATCA  
ACGACAACATACATCGACCGTTACATCGCCCCAGAGTCCAACGTCCTGGT  
CCTGGTCCAGTACCCTGACCGTTCCAAGCTGTACACCGGTAACCCTATCA  
CCATCAAGTCCGTCTCCGACAAGAACCCTTACTCCCGTATCCTGAACGGT  
GACAACATCATCCTGCACATGCTGTACAACTCCCGTAAGTACATGATCAT  
CCGTGACACCGACACCATCTACGCCACCCAGGGTGGTGACTGTTCCCAG  
AACTGTGTCTACGCCCTGAAGCTGCAGTCCAACCTGGGTAACTACGGTAT  
CGGTATCTTCTCCATCAAGAACATCGTCTCCAAGAACAAAGTACTGCTCCC  
AGATCTTCTCCTCCTTCCGTGAGAACACCATGCTGCTGGCCGACATCTAC  
AAGCCTTGGCGTTTCTCCTTCAAGAACGCCTACACTCCTGTCGCCGTCAC  
CAACTACGAGACCAAGCTGCTGTCCACCTCCTCCTTCTGGAAGTTCATCT  
CCCGTGACCCAGGTTGGGTTCGAGtaatagGAATTC

**B: BoNTD(H<sub>o</sub>) encoded protein SEQ ID NO: 12**

MRLKAKVNESFENTMPFNIFS YTNNSLLKDIINEYFNSINDSKILSLQNKKNA  
LVDTSGYNAEVRVGDNVQLNTIYTNDFKLSSSGDKIIVNLNNNIL YSAIYENS  
SVSFWIKSKDLTNSHNEYTIINSIEQNSGWKLCIRNGNIEWILQDVNRKYKS  
LIFDYSESLSHTGYTNKWWFFVTITNNIMGYMKLYINGELKQSQKIEDLDEVK  
LDKTIVFGIDENIDENQMLWIRDFNIFSKELSNEDINIVYEGQILRNVIKDYW  
GNPLKFDTEYYIINDNYIDRYIAPESNVLVLVQYPDRSKLYTGNPITIKSVSD  
KNPYSRILNGDNILHMLYNSRKYMIIRD TDTIYATQGGDCSQNCVYALKLQ  
SNLGNYGIGIFSINKNIVSKNKYCSQIFSSFRENTMLLADIYKPWRFSFKNAYTP  
VAVTNYETKLLSTSSFWKFISRDPGWVE\*\*

FIGURE 7

**A: BoNTE(H<sub>2</sub>) synthetic gene SEQ ID NO: 13**

GAATTCACCAgGGAGAGAGTCAGCAAGAACTAAATTCTATGGTAACTGA  
TACCCTAAATAATAGTATTCCTTTTAAGCTTTCTTCTTATACAGATGATAA  
AATTTTAATTTCTACTTCAACAAGTTCTTCAAGAGAATTAAGTCTTCTTC  
CGTTTTAAACATGAGATACAAGAATGATAAATACGTCGACACTTCCGGT  
TACGACTCCAATATCAACATTAACGGTGACGTGTACAAGTACCCAATA  
ACAAAAACCAATTTCGGTATCTACAACGACAAGCTTACTGAGCTGAACAT  
CTCTCAAAACGACTACATTATCTACGACAACAAGTACAAGAAGTTCTCTA  
TTTCTTTCTGGGTCAGGATTCCTAACTACGACAACAAGATCGTCAACGTT  
AACAACGAGTACACTATCATCAACTGTATGAGAGACAACAACCTCCGGT  
GGAAGGTCTCTCTTAACCACAACGAGATCATTTGGACCTTGCAAGACAA  
CGCAGGTATTAACCAAAAAGTTAGCATTCAACTACGGTAACGCAAACGGT  
ATTTCTGACTACATCAACAAGTGGATTTTCGTCACTATCACTAACGACAG  
ATTAGGTGACTCTAAGCTTTACATTAACGGTAACTTAATCGACCAAAAAGT  
CCATTTTAACTTAGGTAACATTCACGTTTCTGACAACATCTTATTCAAG  
ATCGTTAACTGCAGTTACACCAGATACATTGGCATTAGATACTTCAACAT  
TTTCGACAAGGAGTTAGACGAGACCGAGATTCAAACCTTTATACAGCAAC  
GAACCTAACACCAATATTTTGAAGGACTTCTGGGGTAACTACTTGCTTTA  
CGACAAGGAATACTACTTATTAACGTGTTAAAGCCAAACAACCTTCATT  
GATAGGAGAAAGGATTCTACTTTAAGCATTAAACAACATCAGAAGCACTA  
TTCTTTTAGCTAACAGATTATACTCTGGTATCAAGGTTAAGATCCAAAGA  
GTTAACAACCTCTTCTACTAACGATAACCTTGTTAGAAAGAACGATCAGGT  
CTATATTAACCTTCGTCGCTAGCAAGACTCACTTATTCCCATTATATGCTG  
ATACCGCTACCACCAACAAGGAGAAGACCATCAAGATCTCCTCCTCTGG  
CAACAGATTTAACCAAGTCGTCGTTATGAACTCCGTCGGTAACAACCTGTA  
CCATGAACCTTTAAAAATAATAATGAAATAATATTGGGTTGTTAGGTTTC  
AAGGCAGATACTGTAGTTGCTAGTACTTGGTATTATACCCACATGAGAG  
ATCACACCAACAGCAATGGATGTTTTTGGAACTTTATTTCTGAAGAACAT  
GGATGGCAAGAAAAAataa  
TagGGATCC GCGGCCGC ACGCGT CCCGGG ACTAGT GAATTC

**B: BoNTE(H<sub>2</sub>) encoded protein SEQ ID NO: 14**

MGESQQELNSMVTDTLNNIPFKLSSYTDDKILISYFNKFFKRIKSSSVLNM  
YKNDKYVDTSYDSNININGDVYKYPTNKNQFGIYNDKLTELNISQNDYIIY  
DNKYKNFSISFWVRIPNYDNKIVNVNNEYTIINCMRDNNNGWVKVSLNHNEII  
WTLQDNAGINQKLAFNYGNANGISDYINKWIFVTITNDRLGDSKLYINGNLI  
DQKSILNLGNIHVSNDILFKIVNCSYTRYIGIRYFNIFDKELDETEIQTLYSNEP  
NTNILKDFWGNLYLLYDKEYYLLNVLPNNFIDRRKDSTLSINNIRSTILLANR  
LYSGIKVKIQRVNNSSNDNLVRKNDQVYINFAVASKTHLFLYADTATTNKE  
KTIKISSSGNRFNQVVMNSVGNNTMNFKNNGNIGLLGFKADTVVAST  
WYYTHMRDHTNSNGCFWNFISEEHGWQEK\*\*

AMINO ACID AND DESIGNED NUCLEOTIDE SEQUENCE

[illegible]
$$\infty$$

FIGURE 9

**A: BoNTF(H<sub>c</sub>) synthetic gene SEQ ID NO: 15**

GAATTCACGatgTCCTACACCAACGACAAGATCCTGATCTTGTACTTCAAC  
AAGCTGTACAAGAAGATCAAGGACAACCTCCATCTTGGACATGAGATACG  
AAAACAATAAGTTCATCGACATCTCCGGTTACGGTTCCAACATCTCCATC  
AACGGTGACGTCTACATCTACTCCACCAATAGAAACCAGTTCGGAATCT  
ACTCCTCCAAGCCTTCCGAGGTCAACATCGCTCAGAACAACGACATCAT  
CTACAACGGAAGATACCAGAACTTCTCCATCTCCTTCTGGGTCCGTATCC  
CAAAGTACTTCAACAAGGTCAACCTGAATAACGAGTACACCATCATCGA  
CTGCATCCGTAACAATAACTCCGGATGGAAGATCTCCCTGAACTACAAC  
AAGATCATCTGGACCCTGCAGGACACCGCCGGTAACAATCAGAAGTTGG  
TCTTCAACTACACCCAGATGATCTCCATCTCCGACTACATCAACAAGTGG  
ATCTTCGTCACCATCACCAATAACCGTTTGGGAAACTCCAGAATCTACAT  
CAACGGTAACCTTGATCGACGAGAAGTCCATCTCCAACCTGGGTGACATC  
CACGTCTCCGACAACATTTTGTTCAGATCGTCGGTTGTAACGACACCCG  
TTACGTTCGGGATCCGTTACTTCAAAGTCTTCGACACTGAGTTGGGTAAGA  
CCGAGATCGAGACCTTGTACTCCGACGAGCCTGACCCATCCATCCTGAA  
GGACTTCTGGGGTAACCTGCTGTACAACAAACGTTACTACTTGCTGA  
ACTTGTTGCGTACCGACAAGTCCATCACCCAGAACTCCAACCTTCTTGAAC  
ATCAACCAGCAGAGAGGTGTCTACCAGAAGCCAAACATCTTCTCCAACA  
CCAGATTGTACACCGGAGTCGAGGTCAATTATCAGAAAGAACGGATCTAC  
TGATATTTCCAACACCGATAACTTCGTCAGAAAGAACGATCTGGCTTACA  
TCAACGTTGTGCGACAGAGATGTGCAATACCGTCTGTACGCCGATATCTCT  
ATCGCCAAACCTGAAAAGATCATCAAGCTGATCCGTACCTCTAACTCTA  
ACAACCTCTCTGGGACAAATCATCGTCATGGACTCCATCGGTAATAACTGT  
ACCATGAACTTCCAGAACAACAACGGTGGAACATCGGTTTGTGGGTT  
TCCACTCCAACAACCTTGGTCGCTTCTCCTGCTACTACAACAACATCCGT  
AAGAACACCTCCTCCAACGGTTGCTTCTGGTCCTTCATCTCCAAGGAGCA  
CGGTTGGCAGGAGAACTaatagGAATTC

**B: BoNTF(H<sub>c</sub>) encoded protein SEQ ID NO: 16**

MSYTNDKILILYFNKLYKKIKDNSILDMRYENNKFIDISGYGSNISINGDVYIY  
STNRNQFGIYSSKPSEVNIAQNNDIYNGRYQNFSISFWVRIPKYFNKVNLLN  
EYTIIDCIRNNNSGWKISLNYNKIIWTLQDTAGNNQKLVFNQYTMISISDYIN  
KWIFVTITNNRLGNSRIYINGNLIDEKSISNLGDIHVSDNILFKIVGCNDTRYV  
GIRYFKVFDTELGKTEIETLYSDEPDPSILKDFWGNLYLLYNKRYYLLNLLRT  
DKSITQNSNFLNINQQRGVYQKPNIFSNTRLYTGVEVIIRKNGSTDISNTDNF  
VRKNDLAYINVVD RDVEYRLYADISIAKPEKIIKLIRTSNSNNSLGGIIVMDSI  
GNNCTMNFQNNNGNIGLLGFHSNNLVASSWYNNIRKNTSSNGCFWSFIS  
KEHGWQEN\*\*

FIGURE 10

**A: BoNTG(H<sub>c</sub>) synthetic gene SEQ ID NO: 17**

GAATTCACGatgAAGGACACCATCCTGATCCAGGTCTTCAACAACTACATC  
TCCAACATCTCCTCCAACGCCATCCTGTCCCTGTCCTACCGTGGTGGTCCG  
TCTGATCGACTCCTCCGGTTACGGAGCCACCATGAACGTCGGTTCGACG  
TCATCTTCAACGACATCGGTAACGGTCAGTTCAAGCTGAACAACTCCGA  
GAACTCCAACATCACCGCCCACCAGTCCAAGTTCGTCTGCTACGACTCCA  
TGTTTCGACAACCTTCTCCATCAACTTCTGGGTCCGTACCCCAAAGTACAAC  
AACAACGACATCCAGACCTACCTGCAGAACGAGTACACCATCATCTCCT  
GTATCAAGAACGACTCCGGTTGGAAGGTCTCCATCAAGGGAAACCGTAT  
CATCTGGACCCTGATCGACGTCAACGCCAAGTCCAAGTCCATCTTCTTCG  
AGTACTCCATCAAGGACAACATCTCCGACTACATCAACAAGTGGTTCTCC  
ATCACCATCACCAACGACCGTCTGGGTAACGCCAACATCTACATCAACG  
GTTCCCTGAAGAAGTCCGAGAAGATCCTGAACCTGGACCGTATCAACTC  
CTCCAACGACATCGACTTCAAGCTGATCAACTGTACCGACACCACCAAG  
TTCGTCTGGATCAAGGACTTCAACATCTTCGGTTCGTGAGCTGAACGCCAC  
CGAGGTCTCCTCCCTGTACTGGATCCAGTCCCTCCACCAACACCCTGAAGG  
ACTTCTGGGGAAACCCACTGCGTTACGACACCCAGTACTACCTGTTCAAC  
CAGGGTATGCAGAACATCTACATCAAGTACTTCTCCAAGGCCTCCATGG  
GTGAGACCGCCCCCTCGTACCAACTTCAACAACGCCGCCATCAACTACCA  
GAACCTGTACCTGGGTCTGCGTTTCATCATCAAGAAGGCCTCCAACCTCC  
GTAACATCAACAACGACAACATCGTCCGTGAGGGTGACTACATCTACCT  
GAACATCGACAACATCTCCGACGAGTCCTACCGTGTCTACGTCTGGTCA  
ACTCCAAGGAGATCCAGACCCAGCTGTTCCCTGGCCCCAATCAACGACGA  
CCCTACCTTCTACGACGTCCTGCAGATCAAGAAGTACTACGAGAAGACC  
ACCTACAACCTGTCAGATCCTGTGCGAGAAGGACACCAAGACCTTCGGAC  
TGTTCCGGTATCGGTAAGTTCGTCAAGGACTACGGTTACGTCTGGGACACC  
TACGACAACCTACTTCTGTATCTCCCAGTGGTACCTGCGTTCGTATCTCCGA  
GAACATCAACAAGCTGCGTCTGGGATGTAACCTGGCAGTTCATCCCAGTC  
GACGAGGGTTGGACCGAGtaatagGAATTC

**B: BoNTG(H<sub>c</sub>) encoded protein SEQ ID NO: 18**

MKDTILIQVFNNYISNISSNAILSLSYRGGRLIDSSGYGATMNVGSDVIFNDIG  
NGQFKLNSENSENITAHQSKFVVYDSMFDNFSINFWVRTPKYNNNDIQTYL  
QNEYTIISCIKND SGWKVSIKGNRIIWTLLIDVNAKSKSIFFEYSIKDNISDYINK  
WFSITITNDRLGNANIYINGSLKKSEKILNLDNRINSSNDIDFKLINCTDITTKFV  
WIKDFNIFGRELNATEVSSLYWIQSSTNTLKD FWNPLRYDTQYYL FNQGM  
QNIYIKYFSKASMGETAPRTNFNNAAINYQNL YLGLRFIIKKASNSRNINNDN  
IVREGDYIYLNIDNISDES YRVYVLVNSKEIQTQLFLAPINDDPTFYDVLQIKK  
YYEKT TYNCQILCEKDTKTFGLFGIGKFVKDYG YVWDTYDNYFCISQWYLR  
RISENINKLRLGCNWQFIPVDEGWTE\*\*



FIGURE 11

**A: BoNTA(H<sub>N</sub>) synthetic gene SEQ ID NO: 19**

atgGCTCTGAACGACCTGTGCATCAAAGTTAACAACCTGGGACCTGTTCTTC  
TCCCCGTCTGAAGACAACCTTCACTAACGACCTGAACAAAGGCGAAGAAA  
TCACCTCCGACACTAACATCGAAGCTGCTGAAGAAAACATCTCTCTGGA  
CCTGATCCAGCAGTACTACCTGACTTTCAACTTCGACAACGAACCGGAA  
AACATCTCCATCGAAAACCTGTCTTCCGACATCATCGGTTCAGCTGGAAC  
GATGCCGAACATCGAACGCTTCCCGAACGGCAAGAAATACGAACTGGAC  
AAATACACCATGTTCCACTACCTGCGTGCTCAGGAATTCGAACACGGTA  
AATCTCGTATCGCTCTGACTAACTCCGTTAACGAAGCTCTGCTGAACCCG  
TCTCGCGTTTACACCTTCTTCTCTTCCGACTACGTTAAGAAAGTTAACAA  
AGCTACTGAAGCTGCTATGTTCCCTGGGTTGGGTTGAACAGCTGGTTTACG  
ACTTCACCGACGAAACTTCTGAAGTTTCCACCACTGACAAAATCGCTGAC  
ATCACTATCATCATCCCGTACATCGGCCCGGCTCTGAACATCGGTAAACAT  
GCTGTACAAAGACGACTTCGTTGGTGCTCTGATCTTCTCTGGCGCTGTTA  
TCCTGCTGGAATTCATCCCGGAAATCGCTATCCCGGTTCTGGGTACCTTC  
GCTCTGGTTTCCTACATCGCTAACAAAGTTCTGACTGTTTCAGACCATCGA  
CAACGCTCTGTCTAAACGTAACGAAAAATGGGACGAAGTTTACAAATAC  
ATCGTTACTAACTGGCTGGCTAAAGTTAACACTCAGATCGACCTGATCCG  
TAAGAAGATGAAAGAAGCTCTGGAAAACAGGCTGAAGCTACTAAAGC  
TATCATCAACTACCAGTACAACCAGTACACCGAAGAAGAAAAGAACAAAC  
ATCAACTTCAACATCGATGACCTGTCTCTAAACTGAACGAATCCATCAA  
CAAAGCTATGATCAACATCAACAAATTCCTGAACCAGTGCTCTGTTTCCT  
ACCTGATGAACTCTATGATCCCGTACGGCGTTAAACGCCTGGAAGACTTC  
GACGCTTCCCTGAAAGACGCTCTGCTGAAATACATCCGTGACAACTACG  
GTACTCTGATCGGCCAGGTTGACCGTCTGAAAGACAAGGTTAACAACAC  
CCTGTCTACTGACATCCCGTTCCAGCTGTCCAAATACGTTGACAACCAAGta  
a

**B: BoNTA(H<sub>N</sub>) encoded protein SEQ ID NO: 20**

MALNDLCIKVNNWDLFFSPSEDNFTNDLNKGEEITSDTNIEAAEENISLDLIQ  
QYYLTFNFDNEPENISIENLSSDIIGQLELMPNIERFPNGKKYELDKYTMFHY  
LRAQEFEGHKSRIALTNSVNEALLNPSRVYTFSSDYVKKVNKATEAAMFL  
GWVEQLVYDFTDETSEVSTTDKIADITIIPYIGPALNIGNMLYKDDDFVGALIF  
SGAVILLEFIPIAIPVLGTFALVSYIANKVLTQVQIDNALSKRNEKWDEVYK  
YIVTNWLAKVNTQIDLIRKKMKEALENQAEATKAIINYQYNQYTEEEKNNI  
NFNIDDLSSKLNESINKAMININKFLNQCSVSYLMNSMIPYGVKRLEDFDASL  
KDALLKYIRDNYGTLIGQVDRDKDKVNNTLSTDIPFQLSKYVDNQ\*

FIGURE 12

**A: BoNTB(H<sub>N</sub>) synthetic gene SEQ ID NO: 21**

atgGCTCCAGGAATCTGTATCGACGTCGACAACGAGGACTTGTTCTTCATC  
GCTGACAAGAACTCCTTCTCCGACGACTTGTCCAAGAACGAGAGAATCG  
AGTACAACACCCAGTCCAACATACATCGAGAACGACTTCCCAATCAACGA  
GTTGATCTTGGACACCGACTTGATCTCCAAGATCGAGTTGCCATCCGAGA  
ACACCGAGTCCTTGACTGACTTCAACGTCGACGTCCCAGTCTACGAGAA  
GCAACCAGCTATCAAGAAGATTTTCACCGACGAGAACACCATCTTCCAA  
TACCTGTACTCTCAGACCTTCCCTTTGGACATCAGAGACATCTCCTTGAC  
CTCTTCCTTCGACGACGCCCTGCTGTTCTCCAACAAGGTCTACTCCTTCTT  
CTCCATGGACTACATCAAGACTGCTAACAAGGTCGTCGAGGCCGGTTTG  
TTCGCTGGTTGGGTCAAGCAGATCGTCAACGATTTTCGTCATCGAGGCTAA  
CAAGTCCAACACCATGGACAAGATTGCCGACATCTCCTTGATTGTCCCAT  
ACATCGGTTTGGCCTTGAACGTCGGTAACGAGACCGCCAAGGGTAACTT  
CGAGAACGCTTTCGAGATCGCTGGTGCCTCCATCTTGTTGGAGTTCATCC  
CAGAGTTGTTGATCCCAGTCGTCGGTGCCTTCTTGTTGGAGTCTACATC  
GACAACAAGAACAAGATCATCAAGACCATCGACAACGCTTTGACCAAGA  
GAAACGAGAAGTGGTCCGACATGTACGGTTTGATCGTCGCCCAATGGTT  
GTCCACCGTCAACACCCAATTCTACACCATCAAGGAGGGTATGTACAAG  
GCCTTGAAC TACCAGGCCCAAGCTTTGGAGGAGATCATCAAGTACAGAT  
ACAACATCTACTCCGAGAAGGAGAAGTCCAACATTAACATCGACTTCAA  
CGACATCAACTCCAAGCTGAACGAGGGTATTAACCAGGCCATCGACAAC  
ATCAACAAC TTCATCAACGGTTGTTCCGTCCTCTCTACTTGATGAAGAAGAT  
GATTCCATTGGCCGTCGAGAAGTTGTTGGACTTCGACAACACCCTGAAG  
AAGA ACTTGTTGAAC TACATCGACGAGAACAAAGTTGTACTTGATCGGTT  
CCGCTGAGTACGAGAAGTCCAAGGTCAACAAGTACTTGAAGACCATCAT  
GCCATTGACTTGTCATCTACACCAACGACACCATCTTGATCGAGATGT  
TCtaa

**B: BoNTB(H<sub>N</sub>) encoded protein SEQ ID NO: 22**

MAPGICIDVDNEDLFFIADKNSFSDDL SKNERIEYNTQS NYIENDFPINELILD  
TDLISKIELPSENTESLTDFNVDVPVYEKQPAIKKIFTDENTIFQYLYSQTFFPLD  
IRDISLTSSFDDALLFSNKVYSFFSMDYIKTANKVVEAGLFAGWVKQIVNDF  
VIEANKSNTMDKIADISLIVPYIGLALNVGNETA KGNFENAFEIAGASILLEFI  
PELLIPVVGAFLLSYIDNKNKIIKTIDNALTKRNEKWSDMYGLIVAQWLST  
VNTQFYTIKEGMYKALNYQAQALEEIKYRYNIYSEKEKSNNINIDFNDINSKL  
NEGINQAIDNINNFIN GCSVSYLMKKMIPLAVEKLLDFDNTLKKNLLNYIDE  
NKLYLIGSAEY EKSKVNKYLKTIMPFDLSIYTNDTILIEMF\*

FIGURE 13

**A: BoNTC<sub>1</sub>(H<sub>N</sub>) synthetic gene SEQ ID NO: 23**

atgTCCCTGTACAACAAGACCCTTGACTGTAGAGAGCTGCTGGTGAAGAA  
CACTGACCTGCCATTCATCGGTGACATCAGTGACGTGAAGACTGACATCT  
TCCTGCGTAAGGACATCAACGAGGAGACTGAGGTGATCTACTACCCAGA  
CAACGTGTCAGTAGACCAAGTGATCCTCAGTAAGAACACCTCCGAGCAT  
GGACAACTAGACCTGCTCTACCCTAGTATCGACAGTGAGAGTGAGATCC  
TGCCAGGGGAGAATCAAGTCTTCTACGACAACCGTACCCAGAACGTGGA  
CTACCTGAACTCCTACTACTACCTAGAGTCTCAGAAGCTGAGTGACAAC  
GTGGAGGACTTCACTTTACGCGTTCAATCGAGGAGGCTCTGGACAACA  
GTGCAAAGGTGTACACTTACTTCCCTACCCTGGCTAACAAGGTGAATGCC  
GGTGTGCAAGGTGGTCTGTTCCCTGATGTGGGCAAACGACGTGGTTGAGG  
ACTTCACTACCAACATCCTGCGTAAGGACACACTGGACAAGATCTCAGA  
TGTGTCAGCTATCATCCCCTACATCGGACCCGCACTGAACATCTCCAAC  
CTGTGCGTCGTGGAACTTCACTGAGGCATTTCGCAGTCACTGGTGTCAAC  
ATCCTGCTGGAGGCATTCCCTGAGTTCACAATCCCTGCTCTGGGTGCATT  
CGTGATCTACAGTAAGGTCCAGGAGCGAAACGAGATCATCAAGACCATC  
GACAACTGTCTGGAGCAGAGGATCAAGAGATGGAAGGACTCCTACGAGT  
GGATGATGGGAACGTGGTTGTCCAGGATCATCACCCAGTTCAACAACAT  
CTCCTACCAGATGTACGACTCCCTGAACTACCAGGCAGGTGCAATCAAG  
GCTAAGATCGACCTGGAGTACAAGAAGTACTCCGGAAGCGACAAGGAG  
AACATCAAGAGCCAGGTTGAGAACCTGAAGAACAGTCTGGACGTCAAG  
ATCTCGGAGGCAATGAACAACATCAACAAGTTCATCCGAGAGTGCTCCG  
TCACCTACCTGTTCAAGAACATGCTGCCTAAGGTCATCGACGAGCTGAA  
CGAGTTCGACCGAAACACCAAGGCAAAGCTGATCAACCTGATCGACTCC  
CATAACATCATCCTGGTCGGTGAGGTCGACAAGCTGAAGGCAAAGGTAA  
ACAACAGCTTCCAGAACTaa

**B: BoNTC<sub>1</sub>(H<sub>N</sub>) encoded protein SEQ ID NO: 24**

MSLYNKTLDCRELLVKNTDLPFIGDISDVKTDIFLRKDINEETEVIYYPDNVS  
VDQVILSKNTSEHGQLDLLYPSIDSESEILPGENQVFYDNRTQNVDYLNSYY  
YLESQKLSDNVEDFTFTRSIEEALDNSAKVYTYFPTLANKVNAGVQGGLFL  
MWANDVVEDFTTNILRKDTLDKISDVSAIPYIGPALNISNSVRRGNFTEAFA  
VTGVTILLEAFPEFTIPALGAFVIYSKVQERNEIIKTIDNCLEQRIKRWKDSYE  
WMMGTWLSRIITQFNNISYQMYDSLNYQAGAIKAKIDLEYKKYSGSDKENI  
KSQVENLKNSLDVKISEAMNINIKFIRECSVTYLFKNMLPKVIDELNEFDRN  
TKAKLINLIDSHNIILVGEVDKLKAKVNNSFQN\*

FIGURE 14

**A: BoNTD(H<sub>N</sub>) synthetic gene SEQ ID NO: 25**

atggccAACTCCCGTGACGACTCCACCTGCATCAAGGTCAAGAACAACAGA  
CTGCCATACGTTGCCGACAAGGACTCCATCTCCCAGGAGATCTTCGAGA  
ACAAGATCATCACCGACGAGACCAACGTTCAAACTACTCCGACAAGTT  
CTCTTTGGACGAGTCCATCCTGGACGGTCAGGTCCCAATCAACCCAGAG  
ATCGTCGACCCACTGTTGCCAAACGTCAACATGGAGCCATTGAACCTGCC  
AGGTGAGGAGATCGTCTTCTACGACGACATCACCAAGTACGTCGACTAC  
TTGAACTCCTACTACTACTTGGAGTCTCAAAAGTTGTCTAACAACGTCGA  
GAACATCACCTTGACCACCTCCGTCGAGGAGGCCTTGGGTTACTCTAACA  
AGATCTACACCTTCCTGCCATCCTTGGCTGAGAAGGTTAACAAGGGTGT  
CAAGCTGGTTTGTTCCTGAACTGGGCCAACGAGGTCGTCGAGGACTTCA  
CCACCAACATCATGAAGAAGGACACCCTGGACAAGATCTCCGACGTCTC  
CGTCATCATCCCATAATCGGTCCAGCCTTGAACATCGGTAACCTCCGCCC  
TGAGAGGTAACTTCAACCAGGCCTTCGCCACCGCCGGTGTTCGCCTTCCTG  
CTGGAGGGTTTCCCAGAGTTCACCATCCCAGCCCTGGGTGTCTTCACCTT  
CTACTCCTCCATCCAGGAGAGAGAGAAGATCATCAAGACCATCGAGAAC  
TGCTTGGAGCAGAGAGTCAAGAGATGGAAGGACTCCTACCAGTGGATGG  
TTTCCAACCTGGCTGTCCAGAATCACCAACCAATTCAACCACATCAACTAC  
CAGATGTACGACTCCCTGTCTACCAGGCCGACGCCATCAAGGCCAAGA  
TCGACCTGGAGTACAAGAAGTACTCCGGTTCCGACAAGGAGAACATCAA  
GTCCCAGGTGAGAACCTGAAGAACTCCTTGGACGTCAAGATCTCCGAG  
GCCATGAACAACATCAACAAGTTCATCCGTGAGTGTTCGGTCACCTACCT  
GTTCAAGAACATGCTGCCAAAGGTCATCGACGAGCTGAACAAGTTCGAC  
CTGAGAACCAAGACCGAGCTGATCAACCTGATCGACTCCCACAACATCA  
TCCTGGTTGGTGAGGTTGACtaa

**B: BoNTD(H<sub>N</sub>) encoded protein SEQ ID NO: 26**

MANSRDDSTCIKVKNRLPYVADKDSISQEIFENKIITDETNVQNYSDKFSLD  
ESILDGQVPINPEIVDPLLPNVNMEPLNLPGEIIVFYDDITKYVDYLNSSYYL  
ESQKLSNNVENITLTTSVEEALGYSNKIYTFLPSLAEKVNKG VQAGLFLNWA  
NEVVEDFTTNIMKKDTLDKISDVS VIIPYIGPALNIGNSALRGNFNQAFATAG  
VAFLLLEGFP EFTIPALGVFTFYSSIQEREKI IKT IENCLEQRVKRWKDSYQWM  
VSNWLSRITTQFNHINYQMYDSL SYQADAIKAKIDLEYKKYSGSDKENIKSQ  
VENLKNSLDVKISEAMNNINKFIRECSVTYLFKNMLPKVIDELNKF DLRTKT  
ELINLIDSHNIILVGEVD\*

FIGURE 15

**A: BoNTE(H<sub>N</sub>) synthetic gene SEQ ID NO: 27**

atgTCCATCTGCATCGAGATCAACAACGGTGAGCTGTTCTTCGTGGCTTCC  
GAGAACAGTTACAACGATGACAACATCAACACTCCTAAGGAGATTGACG  
ACACCGTCACTTCTAACAACAACCTACGAAAACGACCTGGACCAGGTCAT  
CCTAAACTTCAACTCCGAGTCCGCCCTGGTCTGTCCGACGAGAAGCTGA  
ACCTGACCATCCAGAACGACGCTTACATCCCAAAGTACGACTCCAACGG  
TACATCCGATATCGAGCAGCATGACGTTAACGAGCTTAACGTCTTCTTCT  
ACTTAGACGCTCAGAAGGTGCCCCGAGGGTGAGAACAACGTCAATCTCAC  
CTCTTCAATTGACACAGCCTTGTTGGAGCAGCCTAAGATCTACACCTTCT  
TCTCCTCCGAGTTCATCAACAACGTCAACAAGCCTGTGCAGGCCGCATTG  
TTCGTAAGCTGGATTTCAGCAGGTGTTAGTAGACTTCACTACTGAGGCTAA  
CCAGAAGTCCACTGTTGACAAGATCGCTGACATCTCCATCGTCGTCCCAT  
ACATCGGTCTGGCTCTGAACATCGGCAACGAGGCACAGAAGGGCAACTT  
CAAGGATGCCCTTGAGTTGTTGGGTGCCGGTATTTTGTGAGTTTCGAAC  
CCGAGCTGCTGATCCCTACCATCCTGGTCTTCACGATCAAGTCCTTCCTG  
GGTTCCTCCGACAACAAGAACAAGGTCATTAAGGCCATCAACAACGCCC  
TGAAGGAGCGTGACGAGAAGTGGAAGGAAGTCTATTCCTTCATCGTCTC  
GAACTGGATGACCAAGATCAACACCCAGTTCAACAAGCGAAAGGAGCA  
GATGTACCAGGCTCTGCAGAACCAGGTCAACGCCATCAAGACCATCATC  
GAGTCCAAGTACAACCTCTACACCCTGGAGGAGAAGAACGAGCTTACCA  
ACAAGTACGATATCAAGCAGATCGAGAACGAGCTGAACCAGAAGGTCTC  
CATCGCCATGAACAACATCGACAGGTTCTTGACCGAGTCCTCCATCTCCT  
ACCTGATGAAGCTCATCAACGAGGTCAAGATCAACAAGCTGCGAGAGTA  
CGACGAGAATGTCAAGACGTACCTGCTGAACTACATCATCCAGCACGGA  
TCCATCCTG<sub>taa</sub>

**B: BoNTE(H<sub>N</sub>) encoded protein SEQ ID NO: 28**

MSICIEINNGELFFVASSENSYNDDNINTPKEIDDTVTSNNNYENDLDQVILNF  
NSESAPGLSDEKLNLTIQNDA YIPKYDSNGTSDIEQHDVNELNVFFYLDAQK  
VPEGENNVLNLTSSIDTALLEQPKIYTFSSSEFINNVNKPVQAALFVSWIQQVL  
VDFTTEANQKSTVDKIADISIVVPYIGLALNIGNEAQKGNFKDALELLGAGIL  
LEFEPELLIPTILVFTIKSFLGSSDNKNKVIKAINNALKERDEKWKEVYSFIVS  
NWMTKINTQFNKRKEQMYQALQNQVNAIKTIIESKYNSYTL EEKNELTNKY  
DIKQIENELNQKVSAMNNIDRFLTESSISYLMKLINEVKINKLREYDENVKT  
YLLNYIIQHGSIL\*

FIGURE 16

**A: BoNTF(H<sub>N</sub>) synthetic gene SEQ ID NO: 29**

atgGCCCCACCACGTCTGTGTATTAGAGTCAACAACCTCAGAATTATTCTTT  
GTCGCTTCCGAGTCAAGCTACAACGAGAACGATATTAACACACCTAAAG  
AGATTGACGATACTACCAACCTAAACAACAACCTACCGGAACAACCTGGA  
TGAGGTTATTTTGGATTACAACCTCACAGACCATCCCTCAAATTTCCAACC  
GTACCTTAAACACTCTTGTCCAAGACAACCTCCTACGTTCCAAGATACGAT  
TCTAACGGTACCTCAGAGATCGAGGAGTATGATGTTGTTGACTTTAACGT  
CTTTTTCTATTTGCATGCCCAGAAGGTGCCAGAAGGTGAAACCAACATCT  
CATTGACTTCTTCCATTGATACCGCCTTGTGGAAGAGTCCAAGGATATC  
TTCTTTTCTTCGGAGTTTATCGATACTATCAACAAGCCTGTCAACGCCGC  
TCTGTTCAATTGATTGGATTAGCAAGGTCATCAGAGATTTTACCACTGAAG  
CTACTCAAAGTCCACTGTTGATAAGATTGCTGACATCTCTTTGATTGTC  
CCCTATGTCGGTCTTGCTTTGAACATCATTATTGAGGCAGAAAAGGGTAA  
CTTTGAGGAGGCTTTTGAATTGTTGGGAGTTGGTATTTTGTGAGTTTG  
TTCCAGAACTTACCATTCTGTCAATTTAGTTTTTACGATCAAGTCCTACA  
TCGATTTCATACGAGAACAAGAATAAAGCAATTAAAGCTATTAACAACCTC  
CTTGATCGAAAGAGAGGCTAAGTGGAAGGAAATCTACTCATGGATTGTA  
TCAAACCTGGCTTACTAGAATTAACACTCAATTTAACAAGAGAAAGGAGC  
AAATGTACCAGGCTCTGCAAAACCAAGTCGATGCTATCAAGACTGCAAT  
TGAATACAAGTACAACAACCTATACTTCCGATGAGAAGAACAGACTTGAA  
TCTGAATACAATATCAACAACATTGAAGAAGAGTTGAACAAGAAAGTTT  
CTTTGGCTATGAAGAATATCGAAAGATTTATGACCGAATCCTCTATCTCT  
TACTTGATGAAGTTGATCAATGAGGCCAAGGTTGGTAAGTTGAAGAAGT  
ACGATAACCACGTTAAGAGCGATCTGCTGAACTACATTCTCGACCACAG  
ATCAATCCTGGGAGAGCAGACAAACGAGCTGAGTGATTGTTACTTCC  
ACTTTGAACTCCTCCATTCCATTTGAGCTTTCT<sub>taa</sub>

**B: BoNTF(H<sub>N</sub>) encoded protein SEQ ID NO: 30**

MAPPRLCIRVNNSELFFVASESSYNENDINTPKEIDDTNLNNNYRNNLDEVI  
LDYNSQTIPQISNRTLNTLVQDNSYVPRYDSNGTSEIEEYDVDFNVFFYLH  
AQKVPEGETNISLTSSIDTALLEESKDIFFSSEFIDTINKPVNAALFIDWISKVIR  
DFTTEATQKSTVDKIADISLIVPYVGLALNIIIEAEKGNFEEAFELLGVGILLEF  
VPELTIPVILVFTIKSYIDSYENKNKAIKAINNSLIEREAKWKEIYSWIVSNWL  
TRINTQFNKRKEQMYQALQNQVDAIKTAIEYKYNNYTSDEKNRLESEYNIN  
NIEEELNKKVSLAMKNIERFMTESISYLMKLINEAKVGKLKKYDNHVKSDL  
LNYILDHRSILGEQTNELSDLVTSTLNSSIPFELS\*

FIGURE 17

**A: BoNTG(H<sub>N</sub>) synthetic gene SEQ ID NO: 31**

atggccAAAAATACCGGTAAATCTGAACAGTGTATTATTGTTAATAATGAG  
GATTTATTTTTTCATAGCTAATAAAGATAGTTTTTCAAAAGATTTAGCTAA  
AGCAGAACTATAGCATATAATACACAAAATAATACTATAGAAAATAAT  
TTTTCTATAGATCAGTTGATTTTAGATAATGATTTAAGCAGTGGCATAGA  
CTTACCAAATGAAAACACAGAACCATTTACAAATTTTGACGACATAGAT  
ATCCCTGTGTATATTAACAATCTGCTTTAAAAAAAATTTTGTGGATGG  
AGATAGCCTTTTTGAATATTTACATGCTCAAACATTTCTTCTAATATAG  
AAAATCTACAACCTAACGAATTCATTAAATGATGCTTTAAGAAATAATAA  
TAAAGTCTATACTTTTTTTTCTACAAACCTTGTTGAAAAAGCTAATACAG  
TTGTAGGTGCTTCACTTTTTGTAACTGGGTAAAAGGAGTAATAGATGAT  
TTTACATCTGAATCCACACAAAAAAGTACTATAGATAAAGTTTCAGATGT  
ATCCATAATTATCCCTATATAGGACCTGCTTTGAATGTAGGAAATGAAA  
CAGCTAAAGAAAATTTTAAAAATGCTTTTGAATAGGTGGAGCCGCTAT  
CTTAATGGAGTTTATTCCAGAAGTATTGTACCTATAGTTGGATTTTTTAC  
ATTAGAATCATATGTAGGAAATAAAGGGCATATTATTATGACGATATCC  
AATGCTTTAAAGAAAAGGGATCAAAAATGGACAGATATGTATGGTTTGA  
TAGTATCGCAGTGGCTCTCAACGGTTAATACTCAATTTTATACAATAAAA  
GAAAGAATGTACAATGCTTTTAAATAATCAATCACAAGCAATAGAAAAAA  
TAATAGAAGATCAATATAATAGATATAGTGAAGAAGATAAAATGAATAT  
TAACATTGATTTTAATGATATAGATTTTAAACTTAATCAAAGTATAAATT  
TAGCAATAAACAATATAGATGATTTTATAAACCAATGTTCTATATCATAT  
CTAATGAATAGAATGATTCCATTAGCTGTAAAAAAGTTAAAAGACTTTG  
ATGATAATCTTAAGAGAGATTTATTGGAGTATATAGATACAAATGAACT  
ATATTTACTTGATGAAGTAAATATTCTAAAATCAAAAGTAAATAGACAC  
CTAAAAGACAGTATACCATTTGATCTTTCCTATATACCtaa

**B: BoNTG(H<sub>N</sub>) encoded protein SEQ ID NO: 32**

MAKNTGKSEQCIIVNNEDLFFIANKDSFSKDLAKAETIAYNTQNNTIENNFSI  
DQLILDNDLSSGIDLPNENTEPFTNFDDIDIPVYIKQSALKKIFVDGDSLFEYL  
HAQTFPSNIENLQLTNSLNDALRNNNKVYTFSTNLVEKANTVVGASLFVN  
WVKGVIDDFTSESTQKSTIDKVS DVSIIPYIGPALNVGNETAKENFKNAFEIG  
GAAILMEFIPELIVPIVGFFTLESYVGNKGHIIMTISNALKKRDQKWTDMYGL  
IVSQWLSTVNTQFYTIKERMYNALNNQSQAIEKIIDQYNYSEEDKMNINI  
DFNDIDFKLNQSINLAINNIDDFINQCSISYLMNRMIPLAVKKLKDFDNLKR  
DLLEYIDTNELYLLDEVNILKSKVNRHLKDSIPFDLSLYT\*

**FIGURE 18**

**A: First BoNTF(Hc) gene: SEQ ID NO: 33**

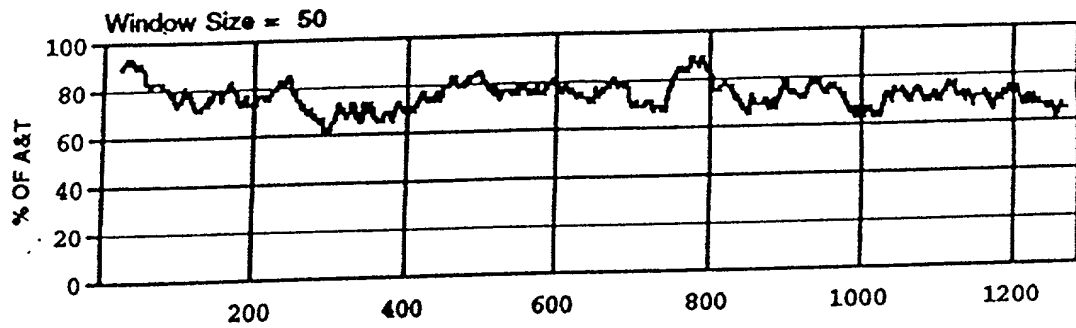
GAATTCACGatgTCTTACACTAACGACAAAATCCTGATCCTGTACTTCAAC  
AAACTGTACAAAAAATCAAAGACAACCTCTATCCTGGACATGCGTTACG  
AAAACAACAAATTCATCGACATCTCTGGCTATGGTTCTAACATCTCTATC  
AACGGTGACGTCTACATCTACTCTACTAACCAGCAACCAGTTCGGTATCTA  
CTCTTCTAAACCGTCTGAAGTAAACATCGCTCAGAACAACGACATCATCT  
ACAACGGTCGTTACCAGAACTTCTCTATCTCTTTCTGGGTTTCGTATCCCG  
AAATACTTCAACAAAGTTAACCTGAACAACGAATACACTATCATCGACT  
GCATCCGTAACAACAACCTCTGGTTGGAAAATCTCTCTGAACTACAACAA  
AATCATCTGGACTCTGCAGGACACTGCTGGTAACAACCAGAACTGGTT  
TTCAACTACACTCAGATGATCTCTATCTCTGACTACATTAATAAATGGAT  
CTTCGTTACTATCACTAACAACCGTCTGGGTAACCTCTCGTATCTACATCA  
ACGGTAACCTGATCGATGAAAAATCTATCTCTAACCTGGGTGACATCCA  
CGTTTCTGACAACATCCTGTTCAAATCGTTGGTTGCAACGACACGCGTT  
ACGTTGGTATCCGTTACTTCAAAGTTTTTCGACACTGAACTGGGTAAAACT  
GAAATCGAACTCTGTACTCTGACGAACCGGACCCGCTATCCTGAAAG  
ACTTCTGGGGTAACTACCTGCTGTACAACAAACGTTACTACCTGCTGAAC  
CTGCTCCGGACTGACAAATCTATCACTCAGAACTCTAACTTCTGAACAT  
CAACCAGCAGCGTGGTGTATTATCAGAAACCTAATATCTTCTCTAACACTC  
GTCTGTACACTGGTGTGAAGTTATCATCCGTAAAAACGGTTCTACTGAC  
ATCTCTAACACTGACAACCTTCGTACGTAAAAACGACCTGGCTTACATCAA  
CGTTGTTGACCGTGACGTTGAATACCGTCTGTACGCTGACATCTCTATCG  
CTAAACCGGAAAAAATCATCAAACCTGATCCGTACTTCTAACTCTAACAA  
CTCTCTGGGTCAGATCATCGTTATGGACTCGATCGGTAACAACCTGCACTA  
TGAACCTCCAGAACAAACACGGTGGTAACATCGGTCTGCTGGGTTTCCA  
CTCTAACAACTGGTTGCTTCTTCATGGTACTACAACAACATCCGTAAAA  
ACACTTCTTCTAACGGTTGCTTCTGGTCTTTCATCTCTAAAGAACACGGTT  
GGCAGGAAAAACtaaGAATTC

**B: BoNTF(Hc) #1 encoded protein: SEQ ID NO: 34**

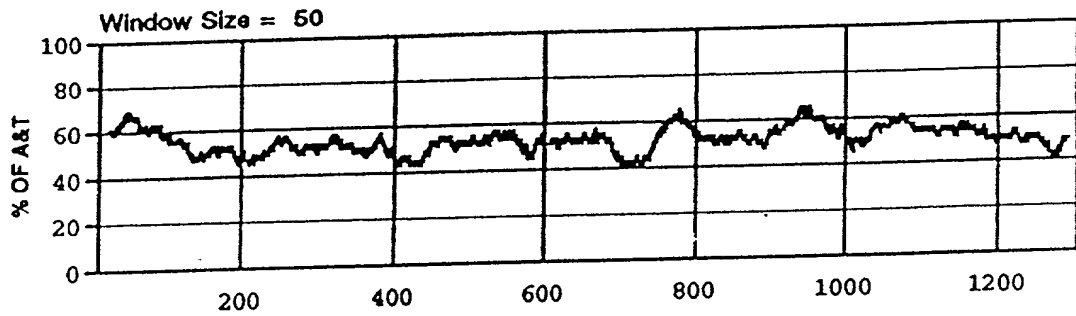
MSYTNDKILLYFNKLYKKIKDNSILDMRYENNKFIDISGYGSNISINGDVYY  
STNRNQFGIYSSKPSEVNIAQNNDIYNGRYQNFSISFWVRIPKYFNKVNLNN  
EYTIIDCIRNNNSGWKISLNYNKIIWTLQDTAGNNQKLVFNYTQMISISDYIN  
KWIFVTITNNRLGNSRIYINGNLIDEKSISNLGDIHVSDNILFKIVGCNDTRYV  
GIRYFKVFDTELKGKTEIETLYSDEPDPSILKDFWGNLYLLYNKRYLLNLLRT  
DKSITQNSNFLNINQQRGVYQKPNIFSNTRLYTGVEVIIRKNGSTDISNTDNF  
VRKNDLAYINVVDRDVEYRLYADISIAKPEKIIKLIRTSNSNSNLGQIIVMDSI  
GNNCTMNFQNNNGGNIGLLGFHSNNLVASSWYYNNIRKNTSSNGCFWSFIS  
KEHGWQEN\*



### A: Natural BoNTF(Hc) Gene



### B: First BoNTF(Hc) Synthetic Gene



### C: Second BoNTF(Hc) Synthetic Gene

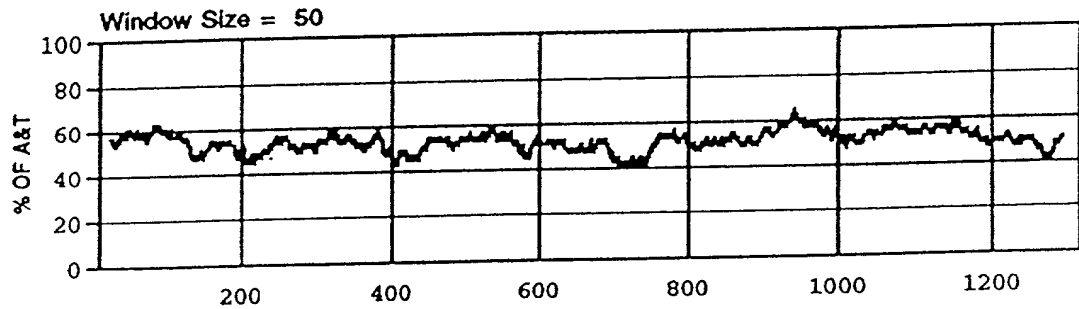


Fig 19

009020-6777960

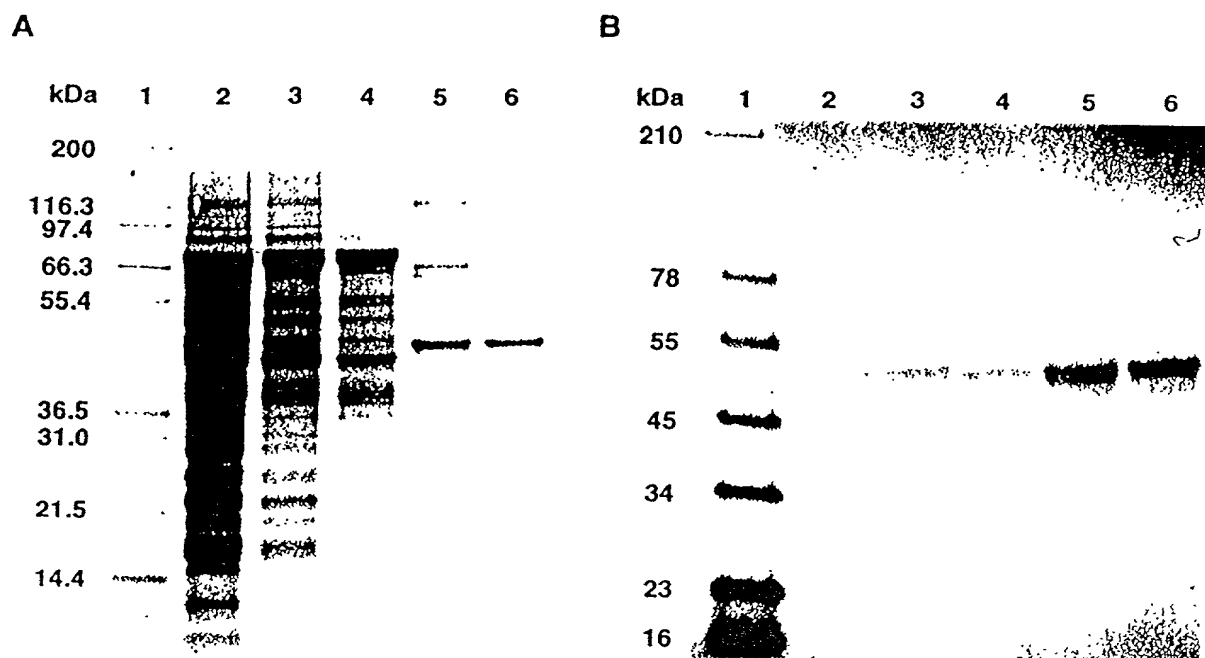


Fig 20

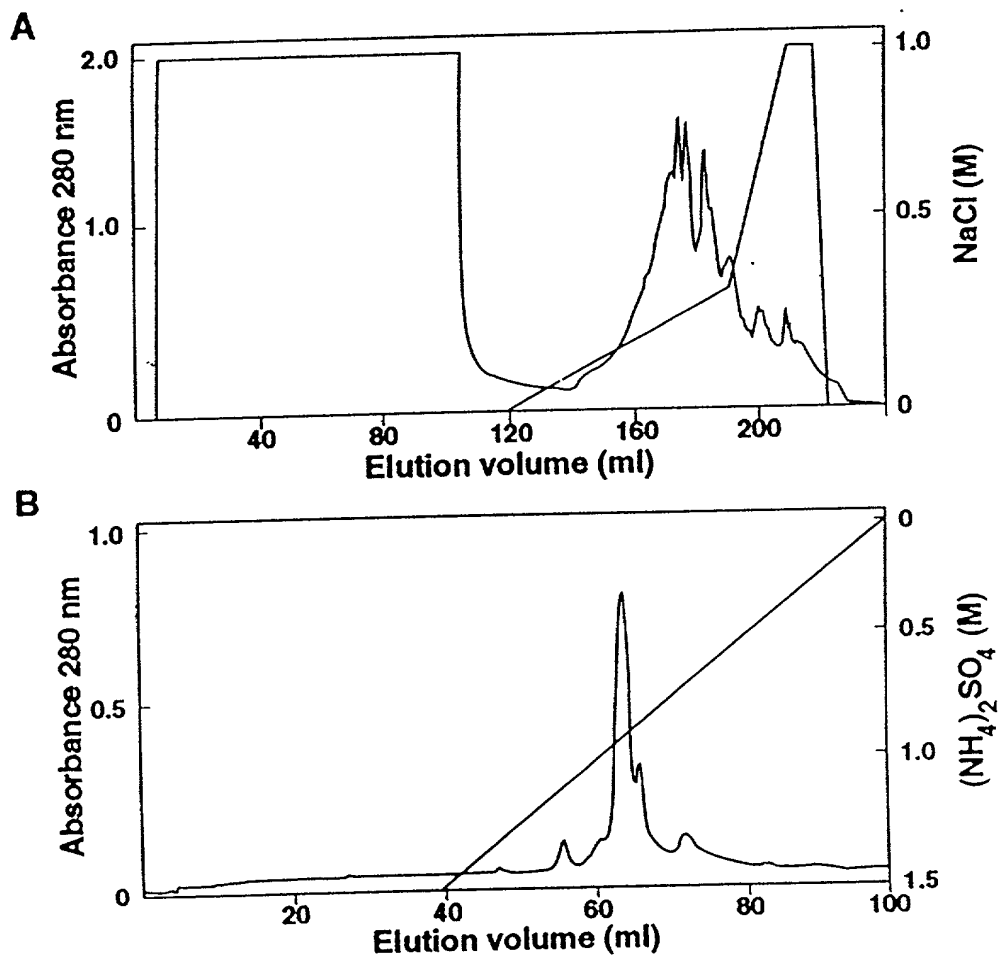


Fig 21

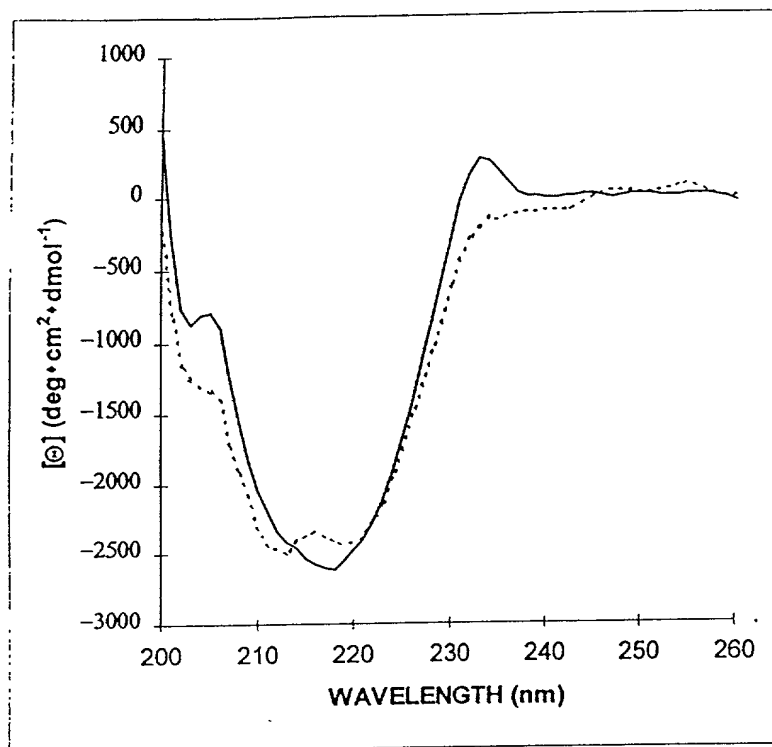


Fig 22

## DECLARATION AND POWER OF ATTORNEY FOR PATENT APPLICATION

As the below named inventor, I hereby declare that:

My residence, post office address and citizenship is as stated below next to my name;

I believe that I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

### RECOMBINANT VACCINE AGAINST BOTULINUM NEUROTOXIN

the specification of which: ☒ is attached hereto.  
☐ was filed on: \_\_\_\_\_  
as Application No.: \_\_\_\_\_  
and was amended on \_\_\_\_\_ (if applicable).

I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above. I acknowledge the duty to disclose information which is material to patentability as defined in 37 C.F.R. § 1.56.

### Prior Foreign Application(s)

I hereby claim foreign priority benefits under Title 35, United States Code, § 119(a)-(d) or § 365(b) of any foreign application(s) for patent or inventor's certificate, or § 365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below any foreign application(s) for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

Country	Application Number	Date of Filing (day, month, year)	Date of Issue (day, month, year)	Priority Claimed	
				Yes <input type="checkbox"/>	No <input type="checkbox"/>
				Yes <input type="checkbox"/>	No <input type="checkbox"/>

### Prior Provisional Application(s)

I hereby claim the benefit under Title 35, United States Code § 119(e) of any United States provisional application(s) listed below:

Application Number	Date of Filing (day, month, year)
60/133,866	May 12, 1999
60/133,868	May 12, 1999
60/133,869	May 12, 1999
60/133,865	May 12, 1999
60/133,873	May 12, 1999
60/133,867	May 12, 1999
60/146,192	July 29, 1999

### Prior United States Application(s)

I hereby claim the benefit under Title 35, United States Code, § 120 of any United States application(s), or § 365(c) of any PCT international application designating the United States of America, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, § 112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, § 1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

Application Number	Date of Filing (day, month, year)	Status - Patented, Pending, Abandoned
PCT/US00/12890	May 12, 2000	Pending

And I hereby appoint, both jointly and severally, as my attorneys with full power of substitution and revocation, to prosecute this application and to transact all business in the Patent and Trademark Office connected herewith the following attorneys, their registration numbers being listed after their names:

Rodger L. Tate, Registration No. 27,399; Scott F. Partridge, Registration No. 28,142; Jerry W. Mills, Registration No. 23,005; Robert Neuner, Registration No. 24,316; James Remenick, Registration No. 36,902; James B. Arpin, Registration No. 33,470; Laurence H. Posorske, Registration No. 34,698; Floyd B. Chapman, Registration No. 40,555; Robert A. King, Registration No. 42,738; David J. Kulik, Registration No. 36,576; Trevor Q. Coddington, Registration No. P46,633; Robert L. Troike, Registration No. 24,183; Jay M. Cantor, Registration No. 19,906; Lori D. Stiffler, Registration No. 36,939; and Jay B. Johnson, Registration No. 38,193.

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine and imprisonment, or both, under 18 U.S.C. § 1001, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Signature \_\_\_\_\_ Date \_\_\_\_\_  
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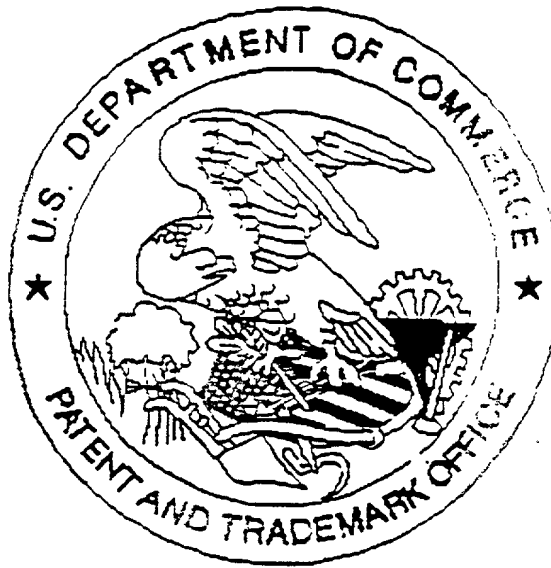
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Ile		ATT	93.00	0.56	Thr		ACT	86.00	0.50
Ile		ATC	72.00	0.44	Thr		ACC	74.00	0.43
Trp		TGG	39.00	1.00	End		TGA	0.00	0.00
End		TGA	0.00	0.00	Cys		TGT	35.00	0.83
Cys		TGT	35.00	0.83	Cys		TGC	7.00	0.17
Cys		TGC	7.00	0.17	End		TAG	1.00	0.20
End		TAG	1.00	0.20	End		TAA	4.00	0.80
Tyr		TAT	18.00	0.12	Tyr		TAT	18.00	0.12
Tyr		TAC	128.00	0.88	Tyr		TAC	128.00	0.88
Leu		TTG	120.00	0.52	Leu		TTA	21.00	0.09
Leu		TTA	21.00	0.09	Phe		TTT	24.00	0.19
Phe		TTT	24.00	0.19	Phe		TTC	104.00	0.81
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Ser		TCG	8.00	0.03	Ser		TCA	14.00	0.07
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Table 1

PICHIA CODON USAGE TABLE

009611449-070600

United States Patent & Trademark Office  
Office of Initial Patent Examination -- Scanning Division



Application deficiencies were found during scanning:

☐ Page(s) \_\_\_\_\_ of \_\_\_\_\_ were not present  
for scanning. (Document title)

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for scanning. (Document title)

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table 1

of figure 20 of drawings